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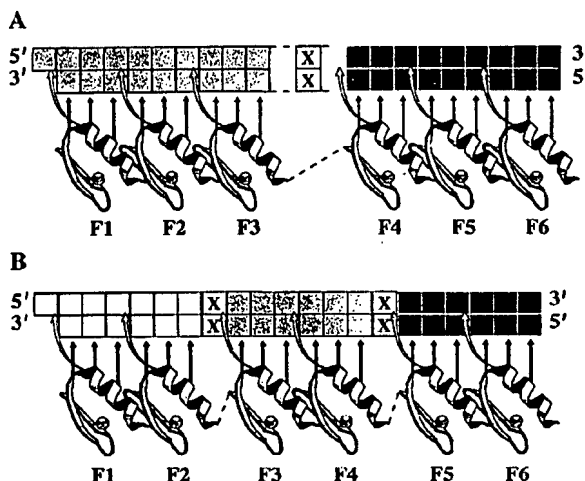
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(54) Title: NUCLEIC ACID BINDING POLYPEPTIDES CHARACTERIZED BY FLEXIBLE LINKERS CONNECTED NUCLEIC ACID BINDING MODULES



(57) Abstract: We describe a method of producing a modified nucleic acid binding polypeptide, the method comprising the steps of: (a) providing a nucleic acid binding polypeptide comprising a plurality of nucleic acid binding modules; (b) selecting a first binding domain consisting of one or two contiguous nucleic acid binding modules; (c) selecting a second binding domain consisting of one or two contiguous nucleic acid binding modules; and (d) introducing a flexible linker sequence to link the first and second binding domains, the flexible linker sequence comprising five or more amino acid residues. Use of structured linkers, alone or in combination with flexible linkers, is also disclosed.



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NUCLEIC ACID BINDING POLYPEPTIDES CHARACTERIZED BY FLEXIBLE LINKERS CONNECTED
NUCLEIC ACID BINDING MODULESFIELD OF THE INVENTION

This invention also relates to linkers for linking together nucleic acid binding polypeptide modules. This invention further relates to nucleic acid binding
5 polypeptides, in particular nucleic acid binding polypeptides capable of binding sequences separated by one or more gaps of varying sizes, and methods for designing such polypeptides.

BACKGROUND OF THE INVENTION

Protein-nucleic acid recognition is a commonplace phenomenon which is
10 central to a large number of biomolecular control mechanisms which regulate the functioning of eukaryotic and prokaryotic cells. For instance, protein-DNA interactions form the basis of the regulation of gene expression and are thus one of the subjects most widely studied by molecular biologists. Many DNA-binding proteins contain independently folded domains for the recognition of DNA, and these domains
15 in turn belong to a large number of structural families, such as the leucine zipper, the "helix-turn-helix" and zinc finger families. Despite the great variety of structural domains, the specificity of the interactions observed to date between protein and DNA most often derives from the complementarity of the surfaces of a protein α -helix and the major groove of DNA (Klug, 1993, *Gene* 135:83-92).

20 Zinc finger proteins are ubiquitous eukaryotic DNA - binding modules first identified in *Xenopus* transcription factor IIIA (TFIIIA). Each zinc finger protein consists of a number of autonomous DNA binding units. For example, the mouse Zif268 zinc finger protein is a protein of 90 amino acid residues belonging to the Cys₂-His₂ zinc family. Zif268 contains three independent zinc finger domains of 24 residues
25 each. Each zinc finger domain ("finger") consists of a single α helix joined to two strands of antiparallel β -sheets and held together via chelation of a zinc ion (Pavletich and Pabo, 1991, *Science* 252, 809-817). Sequence-specific DNA binding is mediated

by residues located on the exposed face of the α helix, which interacts with the major groove of DNA. One zinc finger domain interacts with about three base pairs, so that a number of fingers, which are linked together by linkers, are required to bind a longer DNA sequence. The linkers of various zinc finger proteins have been compared, and a
5 consensus sequence (the "canonical sequence") determined, consisting of four amino acids Gly-Glu-Lys-Pro. This canonical linker is termed the "GEKP linker". However, variants of this sequence are possible, for example, Gly-Gln-Lys-Pro, Gly-Glu-Arg-Pro and Gly-Gln-Arg-Pro.

It has been suggested that the contacts between particular amino acids and
10 DNA base sequence may be described by a simple set of rules. However, current methods for the design and selection of zinc finger modules are not generally capable of producing zinc finger proteins that are capable of binding to any given DNA sequence. This is because certain nucleotide sequences will constitute favourable
binding sites for zinc finger binding. It is known, for example, that DNA sequences
15 which contain G-rich regions are highly specific binding sites for zinc finger proteins. In particular, zinc fingers tend to bind DNA sequences which contain G at every third position with high specificity. On the other hand, with regard to other sequences it will be difficult or impossible to design zinc fingers which bind specifically to that
sequence. Thus, for example, pyrimidine-rich DNA sequences comprise less
20 favourable binding sites for zinc fingers. In order to increase the affinity and specificity of binding, it is therefore desirable to construct zinc fingers which will tolerate gaps between the nucleotide sequences which are contacted by the fingers.

It is known in the prior art to attempt to increase affinity and specificity of zinc finger binding by linking together separate zinc finger domains with a canonical
25 sequence. Thus, Rebar (1997, PhD Thesis, Massachusetts Institute of Technology, Massachusetts, USA) and Shi (1995, PhD Thesis, Johns Hopkins University, Maryland, USA) describe linking additional fingers to a three-finger protein using a GERP linker, and observe a relatively modest increase in affinity. Furthermore, tandem linkage of two three-finger proteins using a canonical linker has been
30 described by Liu et al (1997), *Proc. Natl. Acad. Sci. USA* 94, 5525-5530. The affinity

of binding of this six finger protein is found to be increased approximately 68-74 fold relative to each three-finger peptide, which is a poor result compared to that predicted by theory. A different approach is described by Kim and Pabo (1998, *Proc. Natl. Acad. Sci. USA* 95, 2812-2817), who use structure based design to generate a six-finger
5 construct, using flexible linkers comprising 8 or 11 amino acids to link two three finger peptides (Zif268 and NRE). However, this construct is only capable of spanning a single gap (comprising 0-2 base pairs) in the composite DNA target site. Structure based design has also been used to construct a fusion protein consisting of zinc fingers from Zif268 and the homeodomain from Oct-1 (Pomerantz et al., 1995, *Science* 267,
10 93-6). Thus, in summary, to date, several groups have created six (or nine) -finger fusion peptides to bind long stretches of DNA with high affinity (Kim, J-S. & Pabo, C. O. (1998) *Proc. Natl. Acad. Sci. USA* 95, 2812-2817; Liu, Q., Segal, D. J., Ghiara, J. B. & Barbas, C. F. III (1997) *Proc. Natl. Acad. Sci. USA* 94, 5525-5530; Kamiuchi, T., Abe, E., Imanishi, M., Kaji, T., Nagaoka, M. & Sugiura, Y. (1998) *Biochemistry* 37,
15 13827-13834). However, the affinities of these constructs vary greatly and have generally been far weaker than expected. In addition, all of these peptides have targeted either contiguous DNA sequences, or those containing just one or two nucleotides of unbound DNA.

It is therefore an object of the present invention to provide nucleic acid binding
20 polypeptides which are capable of spanning longer gaps between DNA binding subsites. It is a further object of the invention to provide nucleic acid binding polypeptides which are capable of spanning a greater number of gaps between the DNA binding subsites. It is a yet further object of the invention to provide nucleic acid binding polypeptides which are capable of spanning variable gaps between DNA
25 binding subsites.

SUMMARY OF THE INVENTION

The invention in general provides for the use of linkers to link two or more nucleic acid domains. The linkers according to the invention are non-canonical linkers, which are flexible or structured. According to the invention in its various aspects, we

provide methods of producing a modified nucleic acid binding polypeptide, nucleic acid binding polypeptides as made by such a method, nucleic acid binding polypeptides, nucleic acids encoding such nucleic acid binding polypeptides, host cells transformed with such nucleic acids, pharmaceutical compositions comprising such polypeptides or such nucleic acids, and uses of certain linkers.

According to a first aspect of the invention, we provide a nucleic acid binding proteins comprising nucleic acid binding domains linked by flexible linkers. This aspect of the invention is summarised by the following paragraphs:

We describe a method of producing a modified nucleic acid binding polypeptide, the method comprising the steps of: (a) providing a nucleic acid binding polypeptide comprising a plurality of nucleic acid binding modules; (b) selecting a first binding domain consisting of one or two contiguous nucleic acid binding modules; (c) selecting a second binding domain consisting of one or two contiguous nucleic acid binding modules; and (d) introducing a linker sequence to link the first and second binding domains, the linker sequence comprising five or more amino acid residues. Preferably, the linker sequence is a flexible linker sequence.

Preferably, steps (b) to (d) are repeated. More preferably, in which the binding affinity and/or specificity of the modified polypeptide to a nucleic acid sequence is increased compared to the binding affinity and/or specificity of an unmodified polypeptide.

Preferably, the nucleic acid sequence comprises a sequence which is bound by the unmodified polypeptide. More preferably, the nucleic acid sequence comprises a sequence bound by the unmodified nucleic acid binding polypeptide, into which one or more nucleic acid residues has been inserted. Most preferably, the nucleic acid residue(s) are inserted between target subsites bound by the first and second binding domains of the unmodified polypeptide.

We further describe a method of making a nucleic acid binding polypeptide, the method comprising the steps of: (a) providing a first binding domain and a second binding domain, at least one of the first and second binding domains consisting of one or two nucleic acid binding module(s); and (b) linking the first and second binding domains with a linker sequence comprising five or more amino acid residues.

We further describe a nucleic acid binding polypeptide comprising a first binding domain and a second binding domain linked by a linker sequence comprising five or more amino acid residues, in which at least one of the first and second binding domains consists of one or two nucleic acid binding module(s).

10 The method or polypeptide may be one in which the nucleic acid binding module is a zinc finger of the Cys₂-His₂ type. Preferably, the nucleic acid binding module is selected from the group consisting of naturally occurring zinc fingers and consensus zinc fingers. Most preferably, the nucleic acid binding polypeptide is Zif-GAC.

15 Preferably, the method or polypeptide is such that each of the first and the second binding domains consists of two binding modules. More preferably, the linker sequence comprises between 5 and 8 amino acid residues.

20 Preferably, the linker sequence is provided by insertion of one or more amino acid residues into a canonical linker sequence. The canonical linker sequence may be selected from GEKP, GERP, GQKP and GQRP. Preferably, the linker sequence comprises a sequence selected from: GGEKP, GGQKP, GGSGEKP, GGSGQKP, GGSGGSGEKP, and GGSGGSGQKP.

Preferably, the nucleic acid binding polypeptide comprises a nucleic acid sequence selected from SEQ ID Nos: 22, 23, 24, 25, 26 and 27.

We further describe a nucleic acid binding polypeptide produced by a method as described above, a nucleic acid encoding a nucleic acid binding polypeptide as described above, and a host cell transformed with a nucleic acid as described above.

We further describe a pharmaceutical composition comprising a polypeptide as described above or a nucleic acid as described above, together with a pharmaceutically acceptable carrier.

We further describe a nucleic acid binding polypeptide comprising a repressor domain and a plurality of nucleic acid binding domains, the nucleic acid binding domains being linked by at least one non-canonical linker. The repressor domain may be a transcriptional repressor domain selected from the group consisting of: a KRAB-A domain, an *engrailed* domain and a *snag* domain. Preferably, the nucleic acid binding domains are linked by at least one flexible linker.

According to a second aspect of the invention, we provide nucleic acid binding proteins comprising nucleic acid binding domains linked by *structured linkers*. This aspect of the invention is summarised by the following paragraphs:

We describe a method of producing a modified nucleic acid binding polypeptide, the method comprising the steps of: (a) providing a nucleic acid binding polypeptide comprising a plurality of nucleic acid binding modules; (b) selecting a first binding domain comprising a nucleic acid binding module; (c) selecting a second binding domain comprising a nucleic acid binding module; and (d) introducing a linker sequence comprising a structured linker to link the first and second binding domains.

Preferably, steps (b) to (d) are repeated. More preferably, the binding affinity and/or specificity of the modified polypeptide to a nucleic acid sequence is increased compared to the binding affinity and/or specificity of an unmodified polypeptide.

Preferably, the nucleic acid sequence comprises a sequence which is bound by the unmodified polypeptide. More preferably, the nucleic acid sequence comprises a

sequence bound by the unmodified nucleic acid binding polypeptide, into which one or more nucleic acid residues has been inserted. Most preferably, the nucleic acid residue(s) are inserted between target subsites bound by the first and second binding domains of the unmodified polypeptide. The number of inserted nucleic acid residues
5 may be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or 11 or more.

We further describe a method of making a nucleic acid binding polypeptide, the method comprising the steps of: (a) providing a first binding domain comprising a nucleic acid binding module; (b) providing a second binding domain comprising a nucleic acid binding module; and (c) linking the first and second binding domains with
10 a linker sequence comprising a structured linker.

We further describe provide a non-naturally occurring nucleic acid binding polypeptide comprising a first binding domain comprising a nucleic acid binding module and a second binding domain comprising a nucleic acid binding module, the first and second binding domains being linked by a linker sequence comprising a
15 structured linker.

Preferably, the nucleic acid binding module is a zinc finger of the Cys₂-His₂ type. More preferably, the method or polypeptide is one in which the nucleic acid binding module is selected from the group consisting of naturally occurring zinc fingers and consensus zinc fingers.

20 Preferably, the structured linker comprises an amino acid sequence which is not capable of specifically binding nucleic acid. More preferably, the structured linker is derived from a zinc finger by mutation of one or more of its base contacting residues to reduce or abolish nucleic acid binding activity of the zinc finger. The structured linker may comprise the amino acid sequence of TFIIIA finger IV. Alternatively, the
25 zinc finger is finger 2 of wild type Zif268 mutated at positions -1, 2, 3 and 6.

Preferably, the method or polypeptide is one in which the first or second nucleic acid binding domain is selected from the group consisting of: fingers 1 to 3 of

TFIIIA, GAC and Zif. More preferably, the nucleic acid binding polypeptide comprises substantially the sequence of TF(1-4)-ZIF (SEQ ID NO: 53), GAC-F4-Zif (SEQ ID NO: 54) or Zif-ZnF-GAC (SEQ ID NO: 55). Most preferably, the or each linker sequence comprises one or more further sequence(s), each further sequence
5 comprising a canonical linker sequence, preferably GEKP, GERP, GQKP or GQRP, optionally comprising one or more amino acid sequences inserted into the canonical sequence. The further sequences may be selected from: GGEKP, GGQKP, GGSGEKP, GGSGQKP, GGSGGSGEKP, and GGSGGSQKP.

We further describe a nucleic acid binding polypeptide produced by any of the
10 methods described above, a nucleic acid encoding a nucleic acid binding polypeptide as described above, and a host cell transformed with a nucleic acid as described above. We further describe a pharmaceutical composition comprising a polypeptide as described above or a nucleic acid as described above together with a pharmaceutically acceptable carrier.

15 We further describe the use of a structured linker in a method of making a nucleic acid binding polypeptide. The structured linker may separate first and second nucleic acid binding domains of the nucleic acid binding polypeptide, to enable the polypeptide to bind a nucleic acid target in which subsites bound by respective domains of the polypeptide are separated by one or more nucleic acid residues.

20 We further describe a nucleic acid binding polypeptide comprising a repressor domain and a plurality of nucleic acid binding domains, the nucleic acid binding domains being linked by at least one non-canonical linker. The repressor domain may be a transcriptional repressor domain selected from the group consisting of: a KRAB-A domain, an *engrailed* domain and a *snag* domain. The nucleic acid binding domains
25 may be linked by at least one structured linker.

According to a third aspect of the invention, we provide nucleic acid binding proteins comprising nucleic acid binding domains linked by *structured and flexible*

linkers in any combination. This aspect of the invention is summarised by the following paragraphs:

We describe a method of producing a modified nucleic acid nucleic acid binding polypeptide, the method comprising the steps of: (a) providing a nucleic acid binding polypeptide comprising a plurality of nucleic acid binding modules; (b) selecting a first binding domain consisting of one or two contiguous nucleic acid binding modules; (c) selecting a second binding domain consisting of one or two contiguous nucleic acid binding modules; (d) introducing a first linker sequence to link the first and second binding domains, the linker sequence comprising five or more amino acid residues; (e) selecting a third binding domain comprising a nucleic acid binding module; (f) selecting a fourth binding domain comprising a nucleic acid binding module; and (g) introducing a second linker sequence comprising a structured linker to link the third and fourth binding domains.

Preferably, steps (b) to (d) are repeated. More preferably, steps (e) to (g) are repeated. Preferably, the binding affinity and/or specificity of the modified polypeptide to a nucleic acid sequence is increased compared to the binding affinity and/or specificity of an unmodified polypeptide.

Preferably, the nucleic acid sequence comprises a sequence which is bound by the unmodified polypeptide. More preferably, the nucleic acid sequence comprises a sequence bound by the unmodified nucleic acid binding polypeptide, into which one or more nucleic acid residues has been inserted. Most preferably, the nucleic acid residue(s) are inserted between target subsites bound by the first and second binding domains of the unmodified polypeptide. The number of inserted nucleic acid residues may be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or 11 or more.

We also describe a method of making a nucleic acid binding polypeptide, the method comprising the steps of: (a) providing a first binding domain and a second binding domain, at least one of the first and second binding domains consisting of one or two nucleic acid binding module(s); (b) linking the first and second binding

domains with a first linker sequence comprising five or more amino acid residues; (c) providing a third binding domain comprising a nucleic acid binding module; (d) providing a fourth binding domain comprising a nucleic acid binding module; and (e) linking the third and fourth binding domains with a second linker sequence comprising a structured linker.

We further describe a nucleic acid binding polypeptide comprising a first binding domain consisting of one or two contiguous nucleic acid binding modules and a second binding domain consisting of one or two contiguous nucleic acid binding modules, the first and second binding domains being linked by a first linker sequence comprising five or more amino acid residues; a third binding domain comprising a nucleic acid binding module and a fourth binding domain comprising a nucleic acid binding module, the third and fourth binding domains being linked by a second linker sequence comprising a structured linker.

In the methods and polypeptides described above, the first linker sequence may comprise a flexible linker. Preferably, the nucleic acid binding module is a zinc finger of the Cys₂-His₂ type. More preferably, the nucleic acid binding module is selected from the group consisting of naturally occurring zinc fingers and consensus zinc fingers.

Preferably, each of the first and the second binding domains consists of two binding modules. More preferably, the first linker sequence comprises between 5 and 8 amino acid residues. The first linker sequence may be provided by insertion of one or more amino acid residues into a canonical linker sequence. Preferably, the canonical linker sequence is selected from GEKP, GERP, GQKP and GQRP. More preferably, the first linker sequence comprises a sequence selected from: GGEKP, GGQKP, GGSERGEKP, GGSGQKP, GGSGERGEKP, and GGSGERGEQKP. Most preferably, the nucleic acid binding polypeptide comprises a nucleic acid sequence selected from SEQ ID Nos: 22, 23, 24, 25, 26 and 27.

Preferably, the structured linker comprises an amino acid sequence which is not capable of specifically binding nucleic acid. More preferably, the structured linker comprises the amino acid sequence of TFIIIA finger IV. Alternatively, or in addition, the structured linker is derived from a zinc finger by mutation of one or more of its
5 base contacting residues to reduce or abolish nucleic acid binding activity of the zinc finger. The zinc finger may be finger 2 of wild type Zif268 mutated at positions -1, 2, 3 and 6. Preferably, the third or fourth nucleic acid binding domain is selected from the group consisting of: fingers 1 to 3 of TFIIIA, GAC and Zif.

Preferably, the method or polypeptide as described above is one in which the
10 nucleic acid binding polypeptide comprises substantially the sequence of TF(1-4)-ZIF (SEQ ID NO: 53), GAC-F4-Zif (SEQ ID NO: 54) or Zif-ZnF-GAC (SEQ ID NO: 55). The second linker sequence may comprise one or more further sequence(s), each further sequence comprising a canonical linker sequence, preferably GEKP, GERP, GQKP or GQRP, optionally comprising one or more amino acid sequences inserted
15 into the canonical sequence. The further sequences may be selected from: GGEKP, GGQKP, GGSGEKP, GGSGQKP, GGSGGSGEKP, and GGSGGSGQKP.

We further describe a nucleic acid binding polypeptide produced by a method as described above, a nucleic acid encoding a nucleic acid binding polypeptide as described above, and a host cell transformed with a nucleic acid as described above.

20 We further describe a pharmaceutical composition comprising a polypeptide as described above, or a nucleic acid as described above, together with a pharmaceutically acceptable carrier.

We further describe a nucleic acid binding polypeptide comprising a repressor domain and a plurality of nucleic acid binding domains, the nucleic acid binding
25 domains being linked by at least one flexible linker and by at least one structured linker.

We further describe a nucleic acid binding polypeptide in which the repressor domain is a transcriptional repressor domain selected from the group consisting of: a KRAB-A domain, an *engrailed* domain and a *snag* domain. The nucleic acid binding domains may be linked by at least one flexible linker, or they may be linked by at least one structured linker.

According to a further aspect of the invention, we provide the use of a nucleic acid binding domain comprising two zinc finger modules as a basic unit in the construction of a nucleic acid binding polypeptide.

According to a yet further aspect of the invention, we provide a method of producing a nucleic acid binding polypeptide, the method comprising providing a first and a second nucleic acid binding domain each comprising two zinc finger modules, and linking the first and second nucleic acid binding domains with a structured linker sequence or a flexible linker sequence.

According to a yet further aspect of the invention, we provide the use of an amino acid sequence comprising five or more amino acid residues as a flexible linker to join two or more nucleic acid binding domains comprising two zinc finger modules. According to a yet further aspect of the invention, we provide the use of an amino acid sequence comprising a zinc finger which is not capable of specifically binding nucleic acid, as a structured linker to join two or more nucleic acid binding domains comprising two zinc finger modules. The nucleic acid binding domain is preferably selected from a zinc finger polypeptide library, in which each polypeptide in the library comprises more than one zinc finger and wherein each polypeptide has been at least partially randomised such that the randomisation extends to cover the overlap of a single pair of zinc fingers.

According to a yet further aspect of the invention, we provide a method for producing nucleic acid binding domains comprising two zinc finger modules for use in constructing a nucleic acid binding polypeptide, the method comprising the steps of: (a) providing a zinc finger polypeptide library, in which each polypeptide in the library

comprises more than one zinc finger and wherein each polypeptide has been at least partially randomised such that the randomisation extends to cover the overlap of a single pair of zinc fingers; (b) providing a nucleic acid sequence comprising at least 6 nucleotides; and (c) selecting sequences in the zinc finger library which are capable of
5 binding to the nucleic acid sequence. Preferably, substantially one and a half zinc fingers are randomised in each polypeptide.

According to a yet further aspect of the invention, we provide a nucleic acid binding polypeptide comprising units of zinc finger binding domains linked by flexible and/or structured linkers, each zinc finger binding domain comprising two zinc finger
10 modules.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic diagram showing the construction of the 3x2F and ZIF-GAC zinc finger constructs described here. Step 1: PCR using primer pairs A + a, B + b, C + c, D + d. Step 2: Overlap PCR; template fill-in and amplification with end
15 primers A + b, C + d. Step 3: Digestion with *EagI*, ligation of resulting products; digestion of full-length product with *NdeI* + *NotI*, ligation into pCITE vector.

Figure 2 shows the nucleic acid and amino acid sequence of the ZIF-GAC fusion construct (SEQ ID NO: 21), which is made by joining the third finger of wild-type ZIF to the first finger of the GAC clone using the peptide LRQKDGERP.

20 Figure 3 shows the nucleic acid and amino acid sequence of the 3x2F ZGS construct (SEQ ID NO: 22).

Figure 4 shows the nucleic acid and amino acid sequence of the 3x2F ZGL construct (SEQ ID NO: 23).

Figure 5 shows the nucleic acid and amino acid sequence of the 3x2F ZGXL
25 construct (SEQ ID NO: 24).

Figure 6 shows the nucleic acid and amino acid sequence of the 3x2F ZGSL construct (SEQ ID NO: 25).

Figure 7 shows the nucleic acid and amino acid sequence of the 3x2F ZGLS construct (SEQ ID NO: 26).

5 Figure 8 shows the nucleic acid and amino acid sequence of the 3x1F ZIF construct (SEQ ID NO: 27).

Figure 9A shows results of gel-shift experiments in which the 2x3F ZIF-GAC peptide is tested for binding to either the 9 bp ZIF site alone (target bsA) or the contiguous 18bp ZIF-GAC site (target bsC).

10 Figure 9B shows results of gel-shift experiments in which the 3x2F ZGS and 3x2F ZGL peptides are tested for binding to target bsC. Serial 5-fold dilutions of peptide are indicated by the black triangle (reactions corresponding to left-hand lanes have less peptide than right-hand lanes), and binding site concentration is 0.13nM.

15 Figure 10 shows results of gel-shift experiments in which 3x2F ZGS and 3x2F ZGL peptides are tested for binding to the non-contiguous target sequence, bsD. Serial 5-fold dilutions of peptide are indicated by the black triangle (reactions corresponding to left-hand lanes have less peptide than right-hand lanes), and binding site concentration is 0.13nM.

20 Figure 11 shows results of gel-shift experiments in which 3x2F ZGXL peptide is tested for binding to the contiguous and non-contiguous target sequences bsC, bsD and bsE. Binding of 3x2F ZGS peptide to bsC is also shown for comparison. Serial 5-fold dilutions of peptide are indicated by the black triangle (reactions corresponding to left-hand lanes have less peptide than right-hand lanes), and binding site concentration is 0.13nM.

Figure 12 shows results of gel-shift experiments in which 3x2F ZGSL peptide is tested for binding to the 3x2F ZGXL binding site bsE, the 3x2F ZGL binding site bsD and the 3x2F ZGSL binding site bsF. Serial 5-fold dilutions of peptide are indicated by the black triangle (reactions corresponding to left-hand lanes have less peptide than right-hand lanes), and binding site concentration is 0.10nM.

Figure 13 is a schematic diagram showing the construction of the TFIIIA(F1-4)-ZIF zinc finger construct described here. Step 1: PCR using primer pairs A + a and B + b on wild type TFIIIA and wild type ZIF templates respectively. Step 2: Overlap PCR; template fill-in and amplification with end primers A + b. Step 3: Digestion with *EagI*, ligation of resulting products; digestion of full-length product with *NdeI* + *NotI*, ligation into pCITE vector.

Figure 14 is a schematic diagram showing the construction of the GAC-F4-ZIF zinc finger construct described here. Step 1: PCR using primer pairs C + c and D + d on GAC clone and TFIIIA(F1-4)-ZIF templates respectively. Step 2: Overlap PCR; template fill-in and amplification with end primers C + d. Step 3: Digestion with *EagI*, ligation of resulting products; digestion of full-length product with *NdeI* + *NotI*, ligation into pCITE vector.

Figure 15 shows the nucleic acid and amino acid sequence of the TF(F1-4)-ZIF fusion construct (SEQ ID NO: 53).

Figure 16 shows the nucleic acid and amino acid sequence of the GAC-F4-ZIF construct (SEQ ID NO: 54).

Figure 17 shows the nucleic acid and amino acid sequence of the ZIF-ZnF-GAC construct (SEQ ID NO: 55).

Figure 18 shows results of gel-shift experiments in which the TFIIIA(F1-4)-ZIF peptide is tested for binding to the ZIF binding site (target bsA), the full length TFIIIA(F1-3)-ZIF site with 6 base pairs of intervening DNA, and the TF(F1-3)-ZIF

site with 7 base pairs of intervening DNA. Serial 5-fold dilutions of peptide are indicated by the black triangle (reactions corresponding to left-hand lanes have less peptide than right-hand lanes), and binding site concentration is 0.16nM.

Figure 19 shows results of gel-shift experiments in which the GAC-F4-ZIF peptide is tested for binding to the ZIF binding site (target bsA), and the full length GAC-ZIF site with 8 base pairs of intervening DNA. Serial 5-fold dilutions of peptide are indicated by the black triangle (reactions corresponding to left-hand lanes have less peptide than right-hand lanes), and binding site concentration is 0.10nM.

Figure 20 shows results of gel-shift experiments in which the GAC-F4-ZIF peptide is tested for binding to the ZIF binding site (target bsA), and the GAC-ZIF site with 9 base pairs of intervening DNA. Serial 5-fold dilutions of peptide are indicated by the black triangle (reactions corresponding to left-hand lanes have less peptide than right-hand lanes), and binding site concentration is 0.16nM.

Figure 21 shows results of gel-shift experiments in which the ZIF-ZnF-GAC peptide is tested for binding to the 9 base pair ZIF binding site (target bsA), the full length 18 base pair ZIF-GAC binding site (bsC), and sites with 2, 3, 4 and 5 base pairs between the ZIF and GAC-clone binding sites (labelled respectively Z2G, Z3G, Z4G and Z5G). The nucleotide sequences of Z2G, Z3G, Z4G and Z5G are as follow: Z2G: 5' GCG GAC GCG gtG CGT GGG CG 3', Z3G: 5' GCG GAC GCG agt GCG TGG GCG 3', Z4G: 5' GCG GAC GCG tag tGC GTG GGC G 3', Z5G: 5' GCG GAC GCG cta gtG CGT GGG CG 3'. Serial 5-fold dilutions of peptide are indicated by the black triangle (reactions corresponding to left-hand lanes have less peptide than right-hand lanes), and binding site concentration is 0.10nM.

Figure 22 shows results of gel-shift experiments in which the 2x3F ZIF-GAC peptide is tested for binding to the 9 base pair ZIF binding site (target bsA), the 18 base pair ZIF-GAC binding site (bsC) as well as bs1, bs2, bs3 and bs4, which comprise the ZIF-GAC bsC sequence, but with the three base subsequence recognised by finger 4 of 2x3F ZIF-GAC removed, and 0, 1, 2 or 3 base pairs respectively

inserted in its place. The nucleotide sequences of bs1, bs2, bs3 and bs4 are as follow:
 bs1: GCG GAC GCG TGG GCG, bs2: GCG GAC t GCG TGG GCG, bs3: GCG GAC
 tc GCG TGG GCG and bs4: GCG GAC atc GCG TGG GCG. Serial 5-fold dilutions of
 peptide are indicated by the black triangle (reactions corresponding to left-hand lanes
 5 have less peptide than right-hand lanes), and binding site concentration is 0.01nM.

Figure 23 shows results of gel-shift experiments in which the 3x2F ZGS
 peptide is tested for binding to the 9 base pair ZIF binding site (target bsA), the full
 length 18 base pair ZIF-GAC binding site, and sites bs1, bs2, bs3 and bs4 as indicated
 above for Figure 22. Serial 5-fold dilutions of peptide are indicated by the black
 10 triangle (reactions corresponding to left-hand lanes have less peptide than right-hand
 lanes), and binding site concentration is 0.01nM.

Figure 24. The general structure of the six-finger arrays used in this study and
 potential regions of non-bound DNA marked with an 'X'. (A) 2x3F peptide with 9 bp
 subsites indicated, (B) 3x2F peptides with 6 bp subsites indicated.

15 Figure 25. A selection of DNA binding studies by gel-shift assay. The gels are
 designed to give a comparison between the binding affinities of the 2x3F Zif-GAC and
 3x2F ZGS peptides, and are not necessarily the gels used to quantify binding affinity.
 For example, the amount of 123456 binding site shifted by each peptide is limited by
 protein concentration, rather than Kd. Top: 5-fold dilutions of 2x3F Zif-GAC (from
 20 800 pM-1.3 pM), against 2 pM binding sites. Bottom: 5-fold dilutions of 3x2F ZGS
 (from 700 pM-1.1 pM), against 2 pM binding sites. The proposed binding modes of
 the zinc finger peptides for each binding site is illustrated under each gel image.

Figure 26. A selection of DNA binding studies by gel-shift assay. (A) 5-fold
 dilutions of TF(1-4)-ZIF (from 5.5 nM-9 pM), against 20 pM ZIF binding site; 2 pM
 25 TF6Z and 2 pM TF7Z. (B) 5-fold dilutions of TF(1-3)-flex-ZIF (from 5 nM-8 pM),
 against 20 pM ZIF and 2 pM TF7Z. (C) 5-fold dilutions of ZIF-ZnF-GAC (from 1 nM-
 1.6 pM), against 10 pM ZIF; 0.4 pM ZM; 0.4 pM Z4M; 0.4 pM Z6M and 0.4 pM
 Z8M.

Figure 27 shows the nucleic acid and amino acid sequence of the 2x3F pep11-9 construct.

Figure 28 shows the nucleic acid and amino acid sequence of the 3x2F pep11-9 construct.

5 DETAILED DESCRIPTION OF THE INVENTION

The invention relates to modified nucleic acid binding polypeptides and methods of producing these. A number of different novel nucleic acid binding polypeptides are disclosed. Methods are also disclosed for modifying an existing nucleic acid binding polypeptide comprising a plurality of nucleic acid binding
10 modules. Where the nucleic acid binding polypeptide is provided by modification of an existing nucleic acid binding polypeptide, the binding affinity and/or specificity of the modified polypeptide to a substrate may be as good as, or better, than the corresponding binding affinity and/or specificity of the unmodified or starting nucleic acid to the same substrate.

15 Thus, the methods of our invention allow the production of nucleic acid binding polypeptides with higher binding affinity, or higher binding specificity, or both. As the term is used here, "specificity" means the ability of a nucleic acid binding polypeptide to discriminate between two or more putative nucleic acid targets. The higher its specificity, the less tolerant a nucleic acid binding polypeptide is to changes
20 to the nature of the target, for example, nucleotide insertions, deletions, mutations, inversions, modifications (e.g., methylation, addition of a chemical moiety), etc. A nucleic acid binding polypeptide with high specificity for a target sequence is more discriminatory, and will likely bind to its target with a certain affinity (which may be a high affinity), and less likely to bind another target (which may comprise the target
25 with changes as described above).

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of chemistry, molecular biology, microbiology, recombinant

DNA and immunology, which are within the capabilities of a person of ordinary skill in the art. Such techniques are explained in the literature. See, for example, J. Sambrook, E. F. Fritsch, and T. Maniatis, 1989, *Molecular Cloning: A Laboratory Manual*, Second Edition, Books 1-3, Cold Spring Harbor Laboratory Press; Ausubel, F. M. et al. (1995 and periodic supplements; *Current Protocols in Molecular Biology*, ch. 9, 13, and 16, John Wiley & Sons, New York, N.Y.); B. Roe, J. Crabtree, and A. Kahn, 1996, *DNA Isolation and Sequencing: Essential Techniques*, John Wiley & Sons; J. M. Polak and James O'D. McGee, 1990, *In Situ Hybridization: Principles and Practice*, Oxford University Press; M. J. Gait (Editor), 1984, *Oligonucleotide Synthesis: A Practical Approach*, Irl Press; and, D. M. J. Lilley and J. E. Dahlberg, 1992, *Methods of Enzymology: DNA Structure Part A: Synthesis and Physical Analysis of DNA* Methods in Enzymology, Academic Press. Each of these general texts is herein incorporated by reference.

In a first aspect, we disclose the use of "flexible" linkers to link nucleic acid binding domains consisting of one or two nucleic acid binding modules. Thus, a method according to this aspect of our invention involves selecting binding domains within the nucleic acid binding polypeptide, each domain consisting of one or two nucleic acid binding modules, and linking these by means of a flexible linker sequence comprising five or more amino acid residues. Use of such flexible linkers allows the binding domains to bind to their cognate binding sites in the nucleic acid even when these are separated by one or more gaps, for example 2 gaps, of one, two, three or more nucleic acid residues. Thus, the peptides according to this aspect of the invention are capable of being able to span two short gaps of unbound DNA, while still binding with picomolar affinity to their target sites. In a highly preferred embodiment, the number of nucleic acid binding modules in each of the first and second binding domains is two.

Our invention is also based in part on the surprising discovery that use of linker sequences which adopt a specific conformational structure, rather than flexible linkers, to link two nucleic acid binding modules or domains results in modified nucleic acid binding polypeptides having improved binding characteristics. Such modified

polypeptides are capable of binding nucleic acid targets comprising one or more relatively wide gaps of varying sizes inserted between target subsites.

In a second aspect, therefore, we disclose the use of "structured" linkers to link nucleic acid binding domains comprising at least one nucleic acid binding module.

- 5 Thus, a method according to this aspect of our invention involves selecting binding domains within the nucleic acid binding polypeptide, each domain comprising one or more nucleic acid binding modules, and introducing a linker sequence comprising a structured linker to link the binding domains. By the use of such structured linkers, the binding domains in the modified nucleic acid binding polypeptide are able to bind to
10 their cognate binding sites in the nucleic acid even when these are separated by gaps of five or more nucleic acid residues.

The terms "flexible linker" and "structured linker" will be described and explained in further detail below.

- A nucleic acid binding polypeptide may also be made which comprises a
15 combination of flexible and structured linkers. Therefore, according to a third aspect, a method involves selecting first and second binding domains within the nucleic acid binding polypeptide, each domain consisting of one or two nucleic acid binding modules, and linking these by means of a flexible linker sequence comprising five or more amino acid residues. Further binding domains (third and fourth) within the
20 nucleic acid binding polypeptide are then selected, each domain comprising one or more nucleic acid binding modules, and a linker sequence comprising a structured linker is introduced to link the third and fourth binding domains.

- By "nucleic acid binding module" we mean a unit of peptide sequence which has nucleic acid binding activity. Examples of peptide sequences having nucleic acid
25 binding activity include zinc fingers, leucine zippers, helix-turn-helix domains, and homeodomains. Preferably, the nucleic acid binding polypeptide comprises a zinc finger protein, and the nucleic acid binding modules comprise zinc fingers. A zinc finger binding motif is a structure well known to those in the art and defined in, for

example, Miller *et al.*, (1985) EMBO J. 4:1609-1614; Berg (1988) PNAS (USA) 85:99-102; Lee *et al.*, (1989) Science 245:635-637; see International patent applications WO 96/06166 and WO 96/32475, corresponding to USSN 08/422,107, incorporated herein by reference. More preferably, the polypeptide is a zinc finger
5 protein of the Cys2-His2 class. Accordingly, in preferred embodiments, the nucleic acid binding polypeptides of our invention are zinc finger proteins which comprise one or more structured linkers, or one or more flexible linkers, or a combination of flexible and structured linkers. Where the zinc finger comprises only flexible linkers, the number of zinc fingers in each binding domain linked by a flexible linker is preferably
10 two. The zinc finger as a whole will preferably comprise 2 or more zinc fingers, for example 2, 3, 4, 5 or 6 zinc fingers. More preferably, the polypeptide comprises 6 zinc finger modules.

The nucleic acid binding polypeptides according to the invention need not consist of a uniform number of modules within each linked domain. Thus,
15 polypeptides which comprise linked domains, in which the number of modules within each domain is different from domain to domain, are envisaged. Our invention therefore includes a zinc finger polypeptide comprising any combination of single finger domains and double finger domains, for example, the polypeptide comprising: finger pair - linker - single finger - single finger - finger pair, etc. The nucleic acid
20 binding polypeptides according to this invention furthermore need not consist of only a single type of binding module. For example, hybrid polypeptides comprising more than one type of binding module are envisaged. Such hybrids include fusion proteins comprising: zinc finger and homeodomain, zinc finger and helix-loop-helix, helix-loop-helix and homeodomain, etc. These hybrid polypeptides may be made by
25 modifications of the methods described in, for example, Pomerantz *et al.*, 1995, Science 267, 93-6. Such modifications are regarded as within the skills of the reader. Furthermore, the linkages between the binding domains need not be uniform; they may comprise flexible linkers, structured linkers, or any combination of the two.

According to a further aspect of the invention, a zinc finger domain consisting
30 of two zinc finger modules may be used as a basic unit or building block for the

construction of multifinger nucleic acid binding polypeptides. The two finger module units may be linked by one or more flexible linkers, one or more structured linkers, or a combination of the two. The two finger module units may be produced in a number of ways, by recombinant DNA techniques, or by selection from suitable libraries. We disclose the use of polypeptide and nucleic acid libraries, which comprise or encode zinc finger polypeptides comprising more than one finger, in which the relevant base contacting positions are fully or partially randomised. We show how such libraries, in particular, libraries encoding substantially one and a half fingers, may be used to select zinc finger pairs. We show that such multifinger polypeptides are effective in spanning one or more gaps in the target nucleic acid sequence.

GAP SPANNING AND SELECTIVE BINDING

Nucleic acid binding polypeptides according to our invention are capable of binding to nucleic acids having a number of gaps between binding subsites, and are therefore capable of accommodating more stretches of unbound DNA within target sequences than those previously known. They therefore allow greater flexibility in the choice of potential binding sites. Furthermore, because the nucleic acid binding polypeptides of our invention are capable of spanning a number of gaps of varying stretches, they allow the targeting of the most favourable base contacts while avoiding less favourable nucleotide sequences. By extending the linker sequence between zinc finger pairs, we show that 3x2F peptides are able to accommodate two regions of unbound DNA within their recognition sequence, rather than one, as is the case for 2x3F peptides. Hence, these constructs also allow more flexibility in the selection of DNA target sequences for 'designer' transcription factors.

Furthermore, the nucleic acid binding polypeptides of our invention show a high degree of specificity for their cognate target sites, in that the polypeptides are not tolerant of deletions in the target sequence. We show that by changing the way in which zinc finger arrays are constructed – by linking three 2-finger domains rather than two 3-finger units – far greater selectivity can be achieved through increased sensitivity to mutated or closely related sequences.

Thus, we have found that it is possible for known zinc finger proteins (for example, those comprising canonical linkers and Zif268/NRE as disclosed in WO99/45132) to bind to a subsequence consisting of a cognate target sequence with a target subsite deleted, by one or more of the fingers looping out of the protein-DNA complex. Thus, for example, we have found that a polypeptide consisting of 6 zinc fingers, besides being capable of binding to its cognate 18 base pair target site, is also capable of binding to a 15 base pair subsequence consisting of a 3 base pair deletion of the cognate 18 base pair target site. Thus, a ZIF-ZnF-GAC construct, having the sequences shown in Figure 17, is able to bind to an 18 base pair nucleic acid sequences consisting of the 9 base pair ZIF recognition sequence linked to the 9 base pair GAC recognition sequence. In addition, this zinc finger construct is capable of binding with similar affinity to nucleic acid sequences consisting of 15, 16 or 17 base pairs (i.e., nucleic acid constructs consisting of ZIF and GAC recognition sites, but with 3, 2 or 1 residue removed). Furthermore, this zinc finger construct is also capable of binding with similar affinity to nucleic acid sequences consisting of 19, 20, 21, 22 and 23 base pair nucleic acid sequences comprising the ZIF and GAC recognition sites, separated by 1 to 5 nucleotide stretches. A selection of results from these experiments is shown in Figures 21 and 22 and explained in further detail below in Example 17. Without seeming to be bound by any particular theory, we believe that the versatility of binding of ZIF-ZnF-GAC to such a wide range of sequences is probably due to the middle ZnF finger (structured linker) being capable of looping out of the protein-DNA complex.

Looping out of such unbound fingers may be a general phenomenon. Thus, zinc finger constructs consisting of 2 three finger domains linked by a linker (for example, the 2x3F ZIF-GAC construct described below) are capable of binding nucleic acid sequences consisting of the cognate 18 base pair ZIF-GAC site (i.e., bsC) but with the corresponding target subsite for finger 4 deleted and replaced by 0, 1, 2, or 3 residues, with similar affinity to the full-length site. It would appear that the reason for this is that looping out of one of the fingers in this construct leaves behind two domains still capable of binding nucleic acid (namely a two finger domain and a three finger domain). The strength of binding of these remaining domains is sufficient to allow the entire construct to be bound to the sub-optimal target even with one finger

looped out. Reference is made to Figure 22 and Example 21 below. This phenomenon allows the polydactyl peptides (based on tandemly arrayed three-finger domains) reported in previous studies to bind with relatively high affinity to related DNA sites containing various mutations and deletions. This would effectively mean that these
5 peptides would not exclusively target the desired sequences within complex genomes.

On the other hand, the 3x2F nucleic acid binding polypeptides of our invention (in other words, three pairs of zinc fingers separated by flexible linkers) are only capable of binding these truncated binding sites with greatly reduced affinity, in comparison to their full-length targets. Thus, for example, a 3x2F ZGS construct binds
10 extremely weakly to a nucleic acid sequence consisting of the cognate 18 base pair ZIF-GAC site (i.e., bsC) but with the corresponding target subsite for finger 4 deleted. The affinity of a 3x2F ZGS peptide for such a sequence is similar to the affinity to a 9 base pair ZIF site. Again without seeming to be bound by any particular theory, we believe that this is due to the fact that looping out of this finger leaves behind three
15 separated domains for binding; the fact that these consist of two fingers, one finger and two fingers means that there is insufficient binding affinity for the entire construct to bind with high-affinity to the sub-optimal nucleic acid. The nucleic acid binding polypeptides of our invention therefore exhibit far greater selectivity through increased sensitivity to mutated or closely related sequences. Reference is made to Figure 23 and
20 Example 21 below.

The fact that the constructs according to this aspect of our invention, namely constructs in which pairs of zinc fingers are separated by flexible linkers, appear to be more particular in the targets they will detectably bind to is an additional factor contributing to their specificity.

25 In summary, within a three-finger unit the sub-optimal binding of an individual finger is better compensated for than within a two-finger unit. Therefore, by linking pairs of fingers together (with linkers slightly longer than canonical linkers), a more effective peptide for gene regulation is generated. In other words, the entire zinc finger pair would contribute minimal binding energy to the peptide-DNA complex if one of

the fingers has a sub-optimal binding interaction. The design also improves six-finger peptide – DNA interactions by allowing the peptide to adjust more regularly to the register of the DNA double helix, reducing the strain within the complex, and enhancing the binding affinity. Creating six-finger constructs with two or more extended linker sequences also provides the opportunity to design extended zinc finger peptides that are capable of binding to composite targets with two regions of unbound DNA. The present invention therefore encompasses the use of two finger modules as a basic unit in the design of zinc finger polypeptides.

TARGET SITE

A “target site” is the nucleic acid sequence recognised by a nucleic acid binding polypeptide such as a zinc finger protein. For a zinc finger protein, the length of a target site varies with the number of fingers present, and with the number of sequence specific bonds formed between the protein and the target site. Typically, a two-fingered zinc protein recognises a four to seven base pair target site, a three-fingered zinc finger protein recognises a six to ten base pair target site, and a six-fingered zinc finger protein recognises two adjacent nine to ten base pair target sites. A “subsite” or a “target subsite” is a subsequence of the target site, and corresponds to a portion of the target site recognised by a subunit of the nucleic acid binding polypeptide, for example, a nucleic acid binding domain or module of the nucleic acid binding polypeptide.

FLEXIBLE AND STRUCTURED LINKERS

By “linker sequence” we mean an amino acid sequence that links together two nucleic acid binding modules. For example, in a “wild type” zinc finger protein, the linker sequence is the amino acid sequence lacking secondary structure which lies between the last residue of the α -helix in a zinc finger and the first residue of the β -sheet in the next zinc finger. The linker sequence therefore joins together two zinc fingers. Typically, the last amino acid in a zinc finger is a threonine residue, which caps the α -helix of the zinc finger, while a tyrosine/phenylalanine or another

hydrophobic residue is the first amino acid of the following zinc finger. Accordingly, in a "wild type" zinc finger, glycine is the first residue in the linker, and proline is the last residue of the linker. Thus, for example, in the Zif268 construct, the linker sequence is G(E/Q)(K/R)P.

5 A "flexible" linker is an amino acid sequence which does not have a fixed structure (secondary or tertiary structure) in solution. Such a flexible linker is therefore free to adopt a variety of conformations. An example of a flexible linker is the canonical linker sequence GERP/GEKP/GQRP/GQKP. Flexible linkers are also disclosed in WO99/45132 (Kim and Pabo). By "structured linker" we mean an amino
10 acid sequence which adopts a relatively well-defined conformation when in solution. Structured linkers are therefore those which have a particular secondary and/or tertiary structure in solution.

 Determination of whether a particular sequence adopts a structure may be done in various ways, for example, by sequence analysis to identify residues likely to
15 participate in protein folding, by comparison to amino acid sequences which are known to adopt certain conformations (e.g., known alpha-helix, beta-sheet or zinc finger sequences), by NMR spectroscopy, by X-ray diffraction of crystallised peptide containing the sequence, etc as known in the art.

 The structured linkers of our invention preferably do not bind nucleic acid, but
20 where they do, then such binding is not sequence specific. Binding specificity may be assayed for example by gel-shift as described below.

 The linker may comprise any amino acid sequence that does not substantially hinder interaction of the nucleic acid binding modules with their respective target subsites. Preferred amino acid residues for flexible linker sequences include, but are
25 not limited to, glycine, alanine, serine, threonine proline, lysine, arginine, glutamine and glutamic acid..

The linker sequences between the nucleic acid binding domains preferably comprise five or more amino acid residues. The flexible linker sequences according to our invention consist of 5 or more residues, preferably, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 or more residues. In a highly preferred embodiment of the invention, the flexible linker sequences consist of 5, 7 or 10 residues.

Once the length of the amino acid sequence has been selected, the sequence of the linker may be selected, for example by phage display technology (see for example United States Patent No. 5,260,203) or using naturally occurring or synthetic linker sequences as a scaffold (for example, GQKP and GEKP, see Liu et al., 1997, *Proc. Natl. Acad. Sci. USA* 94, 5525-5530 and Whitlow et al., 1991, *Methods: A Companion to Methods in Enzymology* 2: 97-105). The linker sequence may be provided by insertion of one or more amino acid residues into an existing linker sequence of the nucleic acid binding polypeptide. The inserted residues may include glycine and/or serine residues. Preferably, the existing linker sequence is a canonical linker sequence selected from GEKP, GERP, GQKP and GQRP. More preferably, each of the linker sequences comprises a sequence selected from GGEKP, GGQKP, GGSGEKP, GGSGQKP, GGSGGSGEKP, and GGSGGSGQKP.

Structured linker sequences are typically of a size sufficient to confer secondary or tertiary structure to the linker; such linkers may be up to 30, 40 or 50 amino acids long. In a preferred embodiment, the structured linkers are derived from known zinc fingers which do not bind nucleic acid, or are not capable of binding nucleic acid specifically. An example of a structured linker of the first type is TFIIIA finger IV; the crystal structure of TFIIIA has been solved, and this shows that finger IV does not contact the nucleic acid (Nolte et al., 1998, *Proc. Natl. Acad. Sci. USA* 95, 2938-2943.). An example of the latter type of structured linker is a zinc finger which has been mutagenised at one or more of its base contacting residues to abolish its specific nucleic acid binding capability. Thus, for example, a ZIF finger 2 which has residues -1, 2, 3 and 6 of the recognition helix mutated to serines so that it no longer specifically binds DNA may be used as a structured linker to link two nucleic acid binding domains.

The use of structured or rigid linkers to jump the minor groove of DNA is likely to be especially beneficial in (i) linking zinc fingers that bind to widely separated (>3bp) DNA sequences, and (ii) also in minimising the loss of binding energy due to entropic factors.

- 5 Typically, the linkers are made using recombinant nucleic acids encoding the linker and the nucleic acid binding modules, which are fused via the linker amino acid sequence. The linkers may also be made using peptide synthesis and then linked to the nucleic acid binding modules. Methods of manipulating nucleic acids and peptide synthesis methods are known in the art (see, for example, Maniatis, et al., 1991.
- 10 *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, New York, Cold Spring Harbor Laboratory Press).

NUCLEIC ACID BINDING POLYPEPTIDES

- This invention relates to nucleic acid binding polypeptides. The term “polypeptide” (and the terms “peptide” and “protein”) are used interchangeably to
- 15 refer to a polymer of amino acid residues, preferably including naturally occurring amino acid residues. Artificial analogues of amino acids may also be used in the nucleic acid binding polypeptides, to impart the proteins with desired properties or for other reasons. The term “amino acid”, particularly in the context where “any amino acid” is referred to, means any sort of natural or artificial amino acid or amino acid
- 20 analogue that may be employed in protein construction according to methods known in the art. Moreover, any specific amino acid referred to herein may be replaced by a functional analogue thereof, particularly an artificial functional analogue. Polypeptides may be modified, for example by the addition of carbohydrate residues to form glycoproteins.

- 25 As used herein, “nucleic acid” includes both RNA and DNA, constructed from natural nucleic acid bases or synthetic bases, or mixtures thereof. Preferably, however, the binding polypeptides of the invention are DNA binding polypeptides.

Particularly preferred examples of nucleic acid binding polypeptides are Cys2-His2 zinc finger binding proteins which, as is well known in the art, bind to target nucleic acid sequences via α -helical zinc metal atom co-ordinated binding motifs known as zinc fingers. Each zinc finger in a zinc finger nucleic acid binding protein is responsible for determining binding to a nucleic acid triplet, or an overlapping quadruplet, in a nucleic acid binding sequence. Preferably, there are 2 or more zinc fingers, for example 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 or more zinc fingers, in each binding protein. Advantageously, the number of zinc fingers in each zinc finger binding protein is a multiple of 2.

Thus, in one embodiment, the invention provides a method for preparing a nucleic acid binding polypeptide of the Cys2-His2 zinc finger class capable of binding to a target DNA sequence, in which zinc finger domains comprising one or two, preferably two, zinc finger modules are linked by flexible linkers or structured linkers.

All of the DNA binding residue positions of zinc fingers, as referred to herein, are numbered from the first residue in the α -helix of the finger, ranging from +1 to +9. "-1" refers to the residue in the framework structure immediately preceding the α -helix in a Cys2-His2 zinc finger polypeptide. Residues referred to as "++" are residues present in an adjacent (C-terminal) finger. Where there is no C-terminal adjacent finger, "++" interactions do not operate.

The present invention is in one aspect concerned with the production of what are essentially artificial DNA binding proteins. In these proteins, artificial analogues of amino acids may be used, to impart the proteins with desired properties or for other reasons. Thus, the term "amino acid", particularly in the context where "any amino acid" is referred to, means any sort of natural or artificial amino acid or amino acid analogue that may be employed in protein construction according to methods known in the art. Moreover, any specific amino acid referred to herein may be replaced by a functional analogue thereof, particularly an artificial functional analogue. The nomenclature used herein therefore specifically comprises within its scope functional analogues or mimetics of the defined amino acids.

The α -helix of a zinc finger binding protein aligns antiparallel to the nucleic acid strand, such that the primary nucleic acid sequence is arranged 3' to 5' in order to correspond with the N terminal to C-terminal sequence of the zinc finger. Since nucleic acid sequences are conventionally written 5' to 3', and amino acid sequences N-terminus to C-terminus, the result is that when a nucleic acid sequence and a zinc finger protein are aligned according to convention, the primary interaction of the zinc finger is with the - strand of the nucleic acid, since it is this strand which is aligned 3' to 5'. These conventions are followed in the nomenclature used herein. It should be noted, however, that in nature certain fingers, such as finger 4 of the protein GLI, bind to the + strand of nucleic acid: see Suzuki *et al.*, (1994) NAR 22:3397-3405 and Pavletich and Pabo, (1993) Science 261:1701-1707. The incorporation of such fingers into DNA binding molecules according to the invention is envisaged.

The present invention may be integrated with the rules set forth for zinc finger polypeptide design in our copending European or PCT patent applications having publication numbers; WO 98/53057, WO 98/53060, WO 98/53058, WO 98/53059, describe improved techniques for designing zinc finger polypeptides capable of binding desired nucleic acid sequences. In combination with selection procedures, such as phage display, set forth for example in WO 96/06166, these techniques enable the production of zinc finger polypeptides capable of recognising practically any desired sequence.

Thus, in one embodiment, the invention provides a method for preparing a nucleic acid binding polypeptide of the Cys2-His2 zinc finger class capable of binding to a target DNA sequence, in which zinc finger domains comprising one or two, preferably two, zinc finger modules are linked by flexible linkers or structured linkers, and in which binding to each base of a DNA triplet by an α -helical zinc finger DNA binding module in the polypeptide is determined as follows: if the 5' base in the triplet is G, then position +6 in the α -helix is Arg and/or position ++2 is Asp; if the 5' base in the triplet is A, then position +6 in the α -helix is Gln or Glu and ++2 is not Asp; if the 5' base in the triplet is T, then position +6 in the α -helix is Ser or Thr and position ++2 is Asp; or position +6 is a hydrophobic amino acid other than Ala; if the 5' base in the

triplet is C, then position +6 in the α -helix may be any amino acid, provided that position ++2 in the α -helix is not Asp; if the central base in the triplet is G, then position +3 in the α -helix is His; if the central base in the triplet is A, then position +3 in the α -helix is Asn; if the central base in the triplet is T, then position +3 in the α -helix is Ala, Ser, Ile, Leu, Thr or Val; provided that if it is Ala, then one of the residues at -1 or +6 is a small residue; if the central base in the triplet is 5-meC, then position +3 in the α -helix is Ala, Ser, Ile, Leu, Thr or Val; provided that if it is Ala, then one of the residues at -1 or +6 is a small residue; if the 3' base in the triplet is G, then position -1 in the α -helix is Arg; if the 3' base in the triplet is A, then position -1 in the α -helix is Gln and position +2 is Ala; if the 3' base in the triplet is T, then position -1 in the α -helix is Asn; or position -1 is Gln and position +2 is Ser; if the 3' base in the triplet is C, then position -1 in the α -helix is Asp and Position +1 is Arg; where the central residue of a target triplet is C, the use of Asp at position +3 of a zinc finger polypeptide allows preferential binding to C over 5-meC.

The foregoing represents a set of rules which permits the design of a zinc finger binding protein specific for any given target DNA sequence.

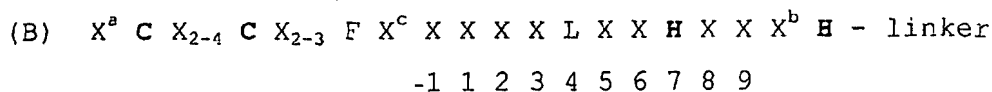
A zinc finger binding motif is a structure well known to those in the art and defined in, for example, Miller *et al.*, (1985) EMBO J. 4:1609-1614; Berg (1988) PNAS (USA) 85:99-102; Lee *et al.*, (1989) Science 245:635-637; see International patent applications WO 96/06166 and WO 96/32475, corresponding to USSN 08/422,107, incorporated herein by reference.

In general, a preferred zinc finger framework has the structure:



where X is any amino acid, and the numbers in subscript indicate the possible numbers of residues represented by X.

In a preferred aspect of the present invention, zinc finger nucleic acid binding motifs may be represented as motifs having the following primary structure:



wherein X (including X^a, X^b and X^c) is any amino acid. X₂₋₄ and X₂₋₃ refer to the presence of 2 or 4, or 2 or 3, amino acids, respectively. The Cys and His residues, which together co-ordinate the zinc metal atom, are marked in bold text and are usually invariant, as is the Leu residue at position +4 in the α -helix. The linker, as noted elsewhere, may comprise a flexible or a structured linker.

Modifications to this representation may occur or be effected without necessarily abolishing zinc finger function, by insertion, mutation or deletion of amino acids. For example it is known that the second His residue may be replaced by Cys (Krizek *et al.*, (1991) J. Am. Chem. Soc. 113:4518-4523) and that Leu at +4 can in some circumstances be replaced with Arg. The Phe residue before X_c may be replaced by any aromatic other than Trp. Moreover, experiments have shown that departure from the preferred structure and residue assignments for the zinc finger are tolerated and may even prove beneficial in binding to certain nucleic acid sequences. Even taking this into account, however, the general structure involving an α -helix co-ordinated by a zinc atom which contacts four Cys or His residues, does not alter. As used herein, structures (A) and (B) above are taken as an exemplary structure representing all zinc finger structures of the Cys2-His2 type.

20 Preferably, X^a is $F/Y-X$ or $P-F/Y-X$. In this context, X is any amino acid. Preferably, in this context X is E, K, T or S. Less preferred but also envisaged are Q, V, A and P. The remaining amino acids remain possible.

Preferably, X₂₋₄ consists of two amino acids rather than four. The first of these amino acids may be any amino acid, but S, E, K, T, P and R are preferred.

25 Advantageously, it is P or R. The second of these amino acids is preferably E, although any amino acid may be used.

Preferably, X^b is T or I. Preferably, X^c is S or T.

Preferably, $X_{2,3}$ is G-K-A, G-K-C, G-K-S or G-K-G. However, departures from the preferred residues are possible, for example in the form of M-R-N or M-R.

As set out above, the major binding interactions occur with amino acids -1, +3
5 and +6. Amino acids +4 and +7 are largely invariant. The remaining amino acids may be essentially any amino acids. Preferably, position +9 is occupied by Arg or Lys. Advantageously, positions +1, +5 and +8 are not hydrophobic amino acids, that is to say are not Phe, Trp or Tyr. Preferably, position ++2 is any amino acid, and preferably serine, save where its nature is dictated by its role as a ++2 amino acid for an
10 N-terminal zinc finger in the same nucleic acid binding molecule.

In a most preferred aspect, therefore, bringing together the above, the invention allows the definition of every residue in a zinc finger DNA binding motif which will bind specifically to a given target DNA triplet.

The code provided by the present invention is not entirely rigid; certain choices
15 are provided. For example, positions +1, +5 and +8 may have any amino acid allocation, whilst other positions may have certain options: for example, the present rules provide that, for binding to a central T residue, any one of Ala, Ser or Val may be used at +3. In its broadest sense, therefore, the present invention provides a very large number of proteins which are capable of binding to every defined target DNA triplet.

20 Preferably, however, the number of possibilities may be significantly reduced. For example, the non-critical residues +1, +5 and +8 may be occupied by the residues Lys, Thr and Gln respectively as a default option. In the case of the other choices, for example, the first-given option may be employed as a default. Thus, the code according to the present invention allows the design of a single, defined polypeptide (a
25 "default" polypeptide) which will bind to its target triplet.

In a further aspect of the present invention, there is provided a method for preparing a DNA binding protein of the Cys2-His2 zinc finger class capable of binding to a target DNA sequence, comprising the steps of: a) selecting a model zinc finger from the group consisting of naturally occurring zinc fingers and consensus zinc
5 fingers; b) mutating at least one of positions -1, +3, +6 (and ++2) of the finger; and c) inserting one or more flexible or structured linkers between zinc finger domains comprising one or two zinc finger modules.

In general, naturally occurring zinc fingers may be selected from those fingers for which the DNA binding specificity is known. For example, these may be the
10 fingers for which a crystal structure has been resolved: namely Zif 268 (Elrod-Erickson *et al.*, (1996) Structure 4:1171-1180), GLI (Pavletich and Pabo, (1993) Science 261:1701-1707), Tramtrack (Fairall *et al.*, (1993) Nature 366:483-487) and YY1 (Houbaviy *et al.*, (1996) PNAS (USA) 93:13577-13582). Preferably, the modified nucleic acid binding polypeptide is derived from Zif 268, GAC, or a Zif-
15 GAC fusion comprising three fingers from Zif linked to three fingers from GAC. By "GAC-clone", we mean a three-finger variant of ZIF268 which is capable of binding the sequence GCGGACGCG, as described in Choo & Klug (1994), *Proc. Natl. Acad. Sci. USA*, 91, 11163-11167.

The naturally occurring zinc finger 2 in Zif 268 makes an excellent starting
20 point from which to engineer a zinc finger and is preferred.

Consensus zinc finger structures may be prepared by comparing the sequences of known zinc fingers, irrespective of whether their binding domain is known. Preferably, the consensus structure is selected from the group consisting of the consensus structure P Y K C P E C G K S F S Q K S D L V K H Q R T H T, and the
25 consensus structure P Y K C S E C G K A F S Q K S N L T R H Q R I H T.

The consensuses are derived from the consensus provided by Krizek *et al.*, (1991) J. Am. Chem. Soc. 113: 4518-4523 and from Jacobs, (1993) PhD thesis, University of Cambridge, UK. In both cases, the linker sequences described above for

joining two zinc finger domains together, namely structured or flexible linkers, can be formed on the ends of the consensus.

When the nucleic acid specificity of the model finger selected is known, the mutation of the finger in order to modify its specificity to bind to the target DNA may be directed to residues known to affect binding to bases at which the natural and
5 desired targets differ. Otherwise, mutation of the model fingers should be concentrated upon residues -1, +3, +6 and ++2 as provided for in the foregoing rules.

In order to produce a binding protein having improved binding, moreover, the rules provided by the present invention may be supplemented by physical or virtual
10 modelling of the protein/DNA interface in order to assist in residue selection.

In a further embodiment, the invention provides a method for producing a zinc finger polypeptide capable of binding to a target DNA sequence, the method comprising: a) providing a nucleic acid library encoding a repertoire of zinc finger domains or modules, the nucleic acid members of the library being at least partially
15 randomised at one or more of the positions encoding residues -1, 2, 3 and 6 of the α -helix of the zinc finger modules; b) displaying the library in a selection system and screening it against a target DNA sequence; c) isolating the nucleic acid members of the library encoding zinc finger modules or domains capable of binding to the target sequence; and d) linking zinc finger domains comprising one or two zinc finger
20 modules with flexible or structured linkers.

Methods for the production of libraries encoding randomised polypeptides are known in the art and may be applied in the present invention. Randomisation may be total, or partial; in the case of partial randomisation, the selected codons preferably encode options for amino acids as set forth in the rules above.

25 Zinc finger polypeptides may be designed which specifically bind to nucleic acids incorporating the base U, in preference to the equivalent base T.

In a further preferred aspect, the invention comprises a method for producing a zinc finger polypeptide capable of binding to a target DNA sequence, the method comprising: a) providing a nucleic acid library encoding a repertoire of zinc finger polypeptides each possessing more than one zinc finger, the nucleic acid members of the library being at least partially randomised at one or more of the positions encoding residues -1, 2, 3 and 6 of the α -helix in a first zinc finger and at one or more of the positions encoding residues -1, 2, 3 and 6 of the α -helix in a further zinc finger of the zinc finger polypeptides; b) displaying the library in a selection system and screening it against a target DNA sequence; d) isolating the nucleic acid members of the library encoding zinc finger polypeptides capable of binding to the target sequence; and e) linking the isolated nucleic acid members with sequences encoding flexible or structured linkers.

In this aspect, the invention encompasses library technology described in our copending International patent application WO 98/53057, incorporated herein by reference in its entirety. WO 98/53057 describes the production of zinc finger polypeptide libraries in which each individual zinc finger polypeptide comprises more than one, for example two or three, zinc fingers; and wherein within each polypeptide partial randomisation occurs in at least two zinc fingers.

This allows for the selection of the "overlap" specificity, wherein, within each triplet, the choice of residue for binding to the third nucleotide (read 3' to 5' on the + strand) is influenced by the residue present at position +2 on the subsequent zinc finger, which displays cross-strand specificity in binding. The selection of zinc finger polypeptides incorporating cross-strand specificity of adjacent zinc fingers enables the selection of nucleic acid binding proteins more quickly, and/or with a higher degree of specificity than is otherwise possible.

Zinc finger binding motifs designed according to the invention may be combined into nucleic acid binding polypeptide molecules having a multiplicity of zinc fingers. Preferably, the proteins have at least two zinc fingers. The presence of at least three zinc fingers is preferred. Nucleic acid binding proteins may be constructed

by joining the required fingers end to end, N-terminus to C-terminus, with flexible or structured linkers. Preferably, this is effected by joining together the relevant nucleic acid sequences which encode the zinc fingers to produce a composite nucleic acid coding sequence encoding the entire binding protein.

- 5 The invention therefore provides a method for producing a DNA binding protein as defined above, wherein the DNA binding protein is constructed by recombinant DNA technology, the method comprising the steps of: preparing a nucleic acid coding sequence encoding a plurality of zinc finger domains or modules defined above, inserting the nucleic acid sequence into a suitable expression vector; and
10 expressing the nucleic acid sequence in a host organism in order to obtain the DNA binding protein. A "leader" peptide may be added to the N-terminal finger. Preferably, the leader peptide is MAEEKP. This aspect of the invention is described in further detail below.

TRANSCRIPTIONAL REGULATION

- 15 According to a further aspect of our invention, we provide a nucleic acid binding polypeptide comprising a repressor domain and a plurality of nucleic acid binding domains, the nucleic acid binding domains being linked by at least one non-canonical linker. The repressor domain is preferably a transcriptional repressor domain selected from the group consisting of: a KRAB-A domain, an *engrailed* domain and a
20 *snag* domain. Such a nucleic acid binding polypeptide may comprise nucleic acid binding domains linked by at least one flexible linker, one or more domains linked by at least one structured linker, or both.

- The nucleic acid binding polypeptides according to our invention may be linked to one or more transcriptional effector domains, such as an activation domain or
25 a repressor domain. Examples of transcriptional activation domains include the VP16 and VP64 transactivation domains of Herpes Simplex Virus. Alternative transactivation domains are various and include the maize C1 transactivation domain sequence (Sainz *et al.*, 1997, Mol. Cell. Biol. 17: 115-22) and P1 (Goff *et al.*, 1992,

Genes Dev. 6: 864-75; Estruch *et al.*, 1994, Nucleic Acids Res. 22: 3983-89) and a number of other domains that have been reported from plants (see Estruch *et al.*, 1994, *ibid*).

Instead of incorporating a transactivator of gene expression, a repressor of gene
5 expression can be fused to the nucleic acid binding polypeptide and used to down
regulate the expression of a gene contiguous or incorporating the nucleic acid binding
polypeptide target sequence. Such repressors are known in the art and include, for
example, the KRAB-A domain (Moosmann *et al.*, Biol. Chem. 378: 669-677 (1997))
the *engrailed* domain (Han *et al.*, Embo J. 12: 2723-2733 (1993)) and the *snag* domain
10 (Grimes *et al.*, Mol Cell. Biol. 16: 6263-6272 (1996)). These can be used alone or in
combination to down-regulate gene expression.

It is known that zinc finger proteins may be fused to transcriptional repression
domains such as the Kruppel-associated box (KRAB) domain to form powerful
repressors. These fusions are known to repress expression of a reporter gene even
15 when bound to sites a few kilobase pairs upstream from the promoter of the gene
(Margolin *et al.*, 1994, PNAS USA 91, 4509-4513). However, because of this, zinc
finger-KRAB fusion proteins are likely to affect the expression of many genes other
than the intended target gene. Thus, the feature of KRAB that it is capable of acting to
repress transcription at a distance is likely to limit its usefulness in gene therapy.
20 However, as zinc fingers of our invention are capable of spanning gaps and may
therefore be engineered to bind specifically to promoter sequences, fusion proteins
comprising KRAB together with zinc fingers of our invention are likely to be effective
in repressing transcription in a specific manner. This could be achieved by designing
zinc fingers to bind to specific promoter sequences, and making use of structured
25 and/or flexible linkers to span non-optimal binding sequences where these are present.
Fusion proteins comprising KRAB and these engineered finger proteins can then be
made by methods known in the art, and used to specifically repress transcription.

NUCLEIC ACIDS ENCODING NUCLEIC ACID BINDING POLYPEPTIDES

The nucleic acid binding polypeptides may be constructed using recombinant techniques as known in the art (Maniatis, et al., 1991. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, New York, Cold Spring Harbor Laboratory Press). Linker sequences may be introduced between the binding domains by
5 restriction enzyme digestion and ligation. For example, zinc finger proteins may be constructed by joining together the relevant nucleic acid coding sequences encoding the zinc fingers to produce a composite coding sequence with the appropriate linkers. Alternatively and preferably, the nucleic acid binding polypeptides are modified by
10 mutagenesis at the existing linker sequences, for example by PCR using mutagenic oligonucleotides. As described in further detail in the Examples, overlap PCR may be used to create chimeric zinc finger proteins having modified linker sequences.

The nucleic acid encoding the nucleic acid binding polypeptide according to the invention can be incorporated into vectors for further manipulation. As used
15 herein, vector (or plasmid) refers to discrete elements that are used to introduce heterologous nucleic acid into cells for either expression or replication thereof. Selection and use of such vehicles are well within the skill of the person of ordinary skill in the art. Many vectors are available, and selection of appropriate vector will depend on the intended use of the vector, i.e. whether it is to be used for DNA
20 amplification or for nucleic acid expression, the size of the DNA to be inserted into the vector, and the host cell to be transformed with the vector. Each vector contains various components depending on its function (amplification of DNA or expression of DNA) and the host cell for which it is compatible. The vector components generally include, but are not limited to, one or more of the following: an origin of replication,
25 one or more marker genes, an enhancer element, a promoter, a transcription termination sequence and a signal sequence. An example of an expression vector is pCITE-4b (Amersham International PLC).

Both expression and cloning vectors generally contain nucleic acid sequence that enable the vector to replicate in one or more selected host cells. Typically in

cloning vectors, this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast and viruses. The origin of replication from the plasmid pBR322 is suitable for most
5 Gram-negative bacteria, the 2 μ plasmid origin is suitable for yeast, and various viral origins (e.g. SV40, polyoma, adenovirus) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors unless these are used in mammalian cells competent for high level DNA replication, such as COS cells.

10 Most expression vectors are shuttle vectors, i.e. they are capable of replication in at least one class of organisms but can be transfected into another class of organisms for expression. For example, a vector is cloned in *E. coli* and then the same vector is transfected into yeast or mammalian cells even though it is not capable of replicating independently of the host cell chromosome. DNA may also be replicated by insertion
15 into the host genome. However, the recovery of genomic DNA encoding the nucleic acid binding polypeptide is more complex than that of exogenously replicated vector because restriction enzyme digestion is required to excise nucleic acid binding polypeptide DNA. DNA can be amplified by PCR and be directly transfected into the host cells without any replication component.

20 Advantageously, an expression and cloning vector may contain a selection gene also referred to as selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that confer
25 resistance to antibiotics and other toxins, e.g. ampicillin, neomycin, methotrexate or tetracycline, complement auxotrophic deficiencies, or supply critical nutrients not available from complex media. As to a selective gene marker appropriate for yeast, any marker gene can be used which facilitates the selection for transformants due to the phenotypic expression of the marker gene. Suitable markers for yeast are, for
30 example, those conferring resistance to antibiotics G418, hygromycin or bleomycin, or

provide for prototrophy in an auxotrophic yeast mutant, for example the URA3, LEU2, LYS2, TRP1, or HIS3 gene.

Since the replication of vectors is conveniently done in *E. coli*, an *E. coli* genetic marker and an *E. coli* origin of replication are advantageously included. These
5 can be obtained from *E. coli* plasmids, such as pBR322, Bluescript™ vector or a pUC plasmid, e.g. pUC18 or pUC19, which contain both *E. coli* replication origin and *E. coli* genetic marker conferring resistance to antibiotics, such as ampicillin.

Suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up nucleic acid binding polypeptide nucleic
10 acid, such as dihydrofolate reductase (DHFR, methotrexate resistance), thymidine kinase, or genes conferring resistance to G418 or hygromycin. The mammalian cell transformants are placed under selection pressure which only those transformants which have taken up and are expressing the marker are uniquely adapted to survive. In the case of a DHFR or glutamine synthase (GS) marker, selection pressure can be
15 imposed by culturing the transformants under conditions in which the pressure is progressively increased, thereby leading to amplification (at its chromosomal integration site) of both the selection gene and the linked DNA that encodes the nucleic acid binding polypeptide. Amplification is the process by which genes in greater demand for the production of a protein critical for growth, together with
20 closely associated genes which may encode a desired protein, are reiterated in tandem within the chromosomes of recombinant cells. Increased quantities of desired protein are usually synthesised from thus amplified DNA.

Expression and cloning vectors usually contain a promoter that is recognised by the host organism and is operably linked to nucleic acid binding polypeptide
25 encoding nucleic acid. Such a promoter may be inducible or constitutive. The promoters are operably linked to DNA encoding the nucleic acid binding polypeptide by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector. Both the native nucleic acid binding polypeptide promoter sequence and many heterologous promoters may be

used to direct amplification and/or expression of nucleic acid binding polypeptide encoding DNA.

Promoters suitable for use with prokaryotic hosts include, for example, the β -lactamase and lactose promoter systems, alkaline phosphatase, the tryptophan (Trp) promoter system and hybrid promoters such as the tac promoter. Their nucleotide sequences have been published, thereby enabling the skilled worker operably to ligate them to DNA encoding nucleic acid binding polypeptide, using linkers or adapters to supply any required restriction sites. Promoters for use in bacterial systems will also generally contain a Shine-Delgarno sequence operably linked to the DNA encoding the nucleic acid binding polypeptide.

Preferred expression vectors are bacterial expression vectors which comprise a promoter of a bacteriophage such as phage λ or T7 which is capable of functioning in the bacteria. In one of the most widely used expression systems, the nucleic acid encoding the fusion protein may be transcribed from the vector by T7 RNA polymerase (Studier et al, *Methods in Enzymol.* 185; 60-89, 1990). In the *E. coli* BL21(DE3) host strain, used in conjunction with pET vectors, the T7 RNA polymerase is produced from the λ -lysogen DE3 in the host bacterium, and its expression is under the control of the IPTG inducible lac UV5 promoter. This system has been employed successfully for over-production of many proteins. Alternatively the polymerase gene may be introduced on a lambda phage by infection with an int-phage such as the CE6 phage which is commercially available (Novagen, Madison, USA). other vectors include vectors containing the lambda PL promoter such as PLEX (Invitrogen, NL) , vectors containing the trc promoters such as pTrcHisXpressTm (Invitrogen) or pTrc99 (Pharmacia Biotech, SE) or vectors containing the tac promoter such as pKK223-3 (Pharmacia Biotech) or PMAL (New England Biolabs, MA, USA).

Moreover, the nucleic acid binding polypeptide gene according to the invention preferably includes a secretion sequence in order to facilitate secretion of the polypeptide from bacterial hosts, such that it will be produced as a soluble native

peptide rather than in an inclusion body. The peptide may be recovered from the bacterial periplasmic space, or the culture medium, as appropriate.

Suitable promoting sequences for use with yeast hosts may be regulated or constitutive and are preferably derived from a highly expressed yeast gene, especially a *Saccharomyces cerevisiae* gene. Thus, the promoter of the TRP1 gene, the ADHI or ADHII gene, the acid phosphatase (PH05) gene, a promoter of the yeast mating pheromone genes coding for the α - or α -factor or a promoter derived from a gene encoding a glycolytic enzyme such as the promoter of the enolase, glyceraldehyde-3-phosphate dehydrogenase (GAP), 3-phospho glycerate kinase (PGK), hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triose phosphate isomerase, phosphoglucose isomerase or glucokinase genes, or a promoter from the TATA binding protein (TBP) gene can be used. Furthermore, it is possible to use hybrid promoters comprising upstream activation sequences (UAS) of one yeast gene and downstream promoter elements including a functional TATA box of another yeast gene, for example a hybrid promoter including the UAS(s) of the yeast PH05 gene and downstream promoter elements including a functional TATA box of the yeast GAP gene (PH05-GAP hybrid promoter). A suitable constitutive PH05 promoter is e.g. a shortened acid phosphatase PH05 promoter devoid of the upstream regulatory elements (UAS) such as the PH05 (-173) promoter element starting at nucleotide -173 and ending at nucleotide -9 of the PH05 gene.

Nucleic acid binding polypeptide gene transcription from vectors in mammalian hosts may be controlled by promoters derived from the genomes of viruses such as polyoma virus, adenovirus, fowlpox virus, bovine papilloma virus, avian sarcoma virus, cytomegalovirus (CMV), a retrovirus and Simian Virus 40 (SV40), from heterologous mammalian promoters such as the actin promoter or a very strong promoter, e.g. a ribosomal protein promoter, and from the promoter normally associated with nucleic acid binding polypeptide sequence, provided such promoters are compatible with the host cell systems.

Transcription of a DNA encoding nucleic acid binding polypeptide by higher eukaryotes may be increased by inserting an enhancer sequence into the vector. Enhancers are relatively orientation and position independent. Many enhancer sequences are known from mammalian genes (e.g. elastase and globin). However, typically one will employ an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270) and the CMV early promoter enhancer. The enhancer may be spliced into the vector at a position 5' or 3' to nucleic acid binding polypeptide DNA, but is preferably located at a site 5' from the promoter.

- 10 Advantageously, a eukaryotic expression vector encoding a nucleic acid binding polypeptide according to the invention may comprise a locus control region (LCR). LCRs are capable of directing high-level integration site independent expression of transgenes integrated into host cell chromatin, which is of importance especially where the nucleic acid binding polypeptide gene is to be expressed in the context of a permanently-transfected eukaryotic cell line in which chromosomal integration of the vector has occurred, or in transgenic animals.

- 20 Eukaryotic vectors may also contain sequences necessary for the termination of transcription and for stabilising the mRNA. Such sequences are commonly available from the 5' and 3' untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding nucleic acid binding polypeptide.

- 25 An expression vector includes any vector capable of expressing nucleic acid binding polypeptide nucleic acids that are operatively linked with regulatory sequences, such as promoter regions, that are capable of expression of such DNAs. Thus, an expression vector refers to a recombinant DNA or RNA construct, such as a plasmid, a phage, recombinant virus or other vector, that upon introduction into an appropriate host cell, results in expression of the cloned DNA. Appropriate expression vectors are well known to those with ordinary skill in the art and include those that are replicable in eukaryotic and/or prokaryotic cells and those that remain episomal or

those which integrate into the host cell genome. For example, DNAs encoding nucleic acid binding polypeptide may be inserted into a vector suitable for expression of cDNAs in mammalian cells, e.g. a CMV enhancer-based vector such as pEVRF (Matthias, et al., (1989) NAR 17, 6418).

5 Particularly useful for practising the present invention are expression vectors that provide for the transient expression of DNA encoding nucleic acid binding polypeptide in mammalian cells. Transient expression usually involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector, and, in turn, synthesises high
10 levels of nucleic acid binding polypeptide. For the purposes of the present invention, transient expression systems are useful e.g. for identifying nucleic acid binding polypeptide mutants, to identify potential phosphorylation sites, or to characterise functional domains of the protein.

Construction of vectors according to the invention employs conventional
15 ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to generate the plasmids required. If desired, analysis to confirm correct sequences in the constructed plasmids is performed in a known fashion. Suitable methods for constructing expression vectors, preparing *in vitro* transcripts, introducing DNA into host cells, and performing analyses for assessing
20 nucleic acid binding polypeptide expression and function are known to those skilled in the art. Gene presence, amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA, dot blotting (DNA or RNA analysis), or *in situ* hybridisation, using an appropriately labelled probe which may be based on a sequence
25 provided herein. Those skilled in the art will readily envisage how these methods may be modified, if desired.

In accordance with another embodiment of the present invention, there are provided cells containing the above-described nucleic acids. Such host cells such as prokaryote, yeast and higher eukaryote cells may be used for replicating DNA and

producing the nucleic acid binding polypeptide. Suitable prokaryotes include eubacteria, such as Gram-negative or Gram-positive organisms, such as *E. coli*, e.g. *E. coli* K-12 strains, DH5a and HB101, or Bacilli. Further hosts suitable for the nucleic acid binding polypeptide encoding vectors include eukaryotic microbes such as

5 filamentous fungi or yeast, e.g. *Saccharomyces cerevisiae*. Higher eukaryotic cells include insect and vertebrate cells, particularly mammalian cells including human cells or nucleated cells from other multicellular organisms. Propagation of vertebrate cells in culture (tissue culture) is a routine procedure and tissue culture techniques are known in the art. Examples of useful mammalian host cell lines are epithelial or

10 fibroblastic cell lines such as Chinese hamster ovary (CHO) cells, NIH 3T3 cells, HeLa cells or 293T cells. The host-cells referred to in this disclosure comprise cells in *in vitro* culture as well as cells that are within a host animal.

DNA may be stably incorporated into cells or may be transiently expressed using methods known in the art. Stably transfected mammalian cells may be prepared

15 by transfecting cells with an expression vector having a selectable marker gene, and growing the transfected cells under conditions selective for cells expressing the marker gene. To prepare transient transfectants, mammalian cells are transfected with a reporter gene to monitor transfection efficiency. To produce such stably or transiently transfected cells, the cells should be transfected with a sufficient amount of the nucleic

20 acid binding polypeptide-encoding nucleic acid to form the nucleic acid binding polypeptide. The precise amounts of DNA encoding the nucleic acid binding polypeptide may be empirically determined and optimised for a particular cell and assay.

Host cells are transfected or, preferably, transformed with the expression or

25 cloning vectors of this invention and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences. Heterologous DNA may be introduced into host cells by any method known in the art, such as transfection with a vector encoding a heterologous DNA by the calcium phosphate coprecipitation technique or by

30 electroporation. Numerous methods of transfection are known to the skilled worker in

the field. Successful transfection is generally recognised when any indication of the operation of this vector occurs in the host cell. Transformation is achieved using standard techniques appropriate to the particular host cells used.

5 Incorporation of cloned DNA into a suitable expression vector, transfection of eukaryotic cells with a plasmid vector or a combination of plasmid vectors, each encoding one or more distinct genes or with linear DNA, and selection of transfected cells are well known in the art (see, e.g. Sambrook et al., 1989 Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press).

10 Transfected or transformed cells are cultured using media and culturing methods known in the art, preferably under conditions, whereby the nucleic acid binding polypeptide encoded by the DNA is expressed. The composition of suitable media is known to those in the art, so that they can be readily prepared. Suitable culturing media are also commercially available.

15 The binding affinity of the nucleic acid binding polypeptides according to our invention may be improved by randomising the polypeptides and selecting for improved binding. Methods for randomisation are disclosed in, for example, WO96/06166. Thus, zinc finger molecules designed according to the invention may be subjected to limited randomisation and subsequent selection, such as by phage display, in order to optimise the binding characteristics of the molecule.

20 The sequences of zinc finger binding motifs may be randomised at selected sites and the randomised molecules obtained may be screened and selected for molecules having the most advantageous properties. Generally, those molecules showing higher affinity and/or specificity of the target nucleic acid sequence are selected. Mutagenesis and screening of target nucleic acid molecules may be achieved
25 by any suitable means. Preferably, the mutagenesis is performed at the nucleic acid level, for example by synthesising novel genes encoding mutant proteins and expressing these to obtain a variety of different proteins. Alternatively, existing genes

can be themselves mutated, such by site-directed or random mutagenesis, in order to obtain the desired mutant genes.

Instead of, or in addition to, randomisation of the zinc finger sequence, a particular amino acid sequence may be chosen on the basis of rules which determine the optimal sequence for binding to any particular nucleic acid sequence. Such rules
5 are disclosed, for example, in our International Application PCT/GB98/01516 (published as WO98/53060).

Mutations may be performed by any method known to those of skill in the art. Preferred, however, is site-directed mutagenesis of a nucleic acid sequence encoding the protein of interest. A number of methods for site-directed mutagenesis are known
10 in the art, from methods employing single-stranded phage such as M13 to PCR-based techniques (see "PCR Protocols: A guide to methods and applications", M.A. Innis, D.H. Gelfand, J.J. Sninsky, T.J. White (eds.). Academic Press, New York, 1990). The commercially available Altered Site II Mutagenesis System (Promega) may be
15 employed, according to the directions given by the manufacturer.

Screening of the proteins produced by mutant genes is preferably performed by expressing the genes and assaying the binding ability of the protein product. A simple and advantageously rapid method by which this may be accomplished is by phage display, in which the mutant polypeptides are expressed as fusion proteins with the
20 coat proteins of filamentous bacteriophage, such as the minor coat protein pII of bacteriophage M13 or gene III of bacteriophage Fd, and displayed on the capsid of bacteriophage transformed with the mutant genes. The target nucleic acid sequence is used as a probe to bind directly to the protein on the phage surface and select the phage possessing advantageous mutants, by affinity purification. The phage are then
25 amplified by passage through a bacterial host, and subjected to further rounds of selection and amplification in order to enrich the mutant pool for the desired phage and eventually isolate the preferred clone(s). Detailed methodology for phage display is known in the art and set forth, for example, in US Patent 5,223,409; Choo and Klug, (1995) *Current Opinions in Biotechnology* 6:431-436; Smith, (1985) *Science*

228:1315-1317; and McCafferty *et al.*, (1990) *Nature* 348:552-554; all incorporated herein by reference. Vector systems and kits for phage display are available commercially, for example from Pharmacia.

Binding affinity may also be assayed by means of a gel-shift assay, in which
5 the mobility of a substrate in a gel is reduced in the presence of binding by a polypeptide. The nucleic acid substrate is labelled by, for example, ^{32}P , for the band-shift to be easily visualised.

USES

Nucleic acid binding polypeptides according to the invention may be employed
10 in a wide variety of applications, including diagnostics and as research tools. Advantageously, they may be employed as diagnostic tools for identifying the presence of nucleic acid molecules in a complex mixture. Nucleic acid binding molecules according to the invention may be used to differentiate single base pair changes in target nucleic acid molecules. In a preferred embodiment, the nucleic acid
15 binding molecules of the invention can be incorporated into an ELISA assay. For example, phage displaying the molecules of the invention can be used to detect the presence of the target nucleic acid, and visualised using enzyme-linked anti-phage antibodies.

Further improvements to the use of zinc finger phage for diagnosis can be
20 made, for example, by co-expressing a marker protein fused to the minor coat protein (gVIII) of bacteriophage. Since detection with an anti-phage antibody would then be obsolete, the time and cost of each diagnosis would be further reduced. Depending on the requirements, suitable markers for display might include the fluorescent proteins (A. B. Cubitt, *et al.*, (1995) *Trends Biochem Sci.* 20, 448-455; T. T. Yang, *et al.*, (1996)
25 *Gene* 173, 19-23), or an enzyme such as alkaline phosphatase which has been previously displayed on gIII (J. McCafferty, R. H. Jackson, D. J. Chiswell, (1991) *Protein Engineering* 4, 955-961) Labelling different types of diagnostic phage with distinct markers would allow multiplex screening of a single nucleic acid sample.

Nevertheless, even in the absence of such refinements, the basic ELISA technique is reliable, fast, simple and particularly inexpensive. Moreover it requires no specialised apparatus, nor does it employ hazardous reagents such as radioactive isotopes, making it amenable to routine use in the clinic. The major advantage of the protocol is that it
5 obviates the requirement for gel electrophoresis, and so opens the way to automated nucleic acid diagnosis.

Polypeptides made according to the invention may be employed in the manufacture of chimeric restriction enzymes, in which a nucleic acid cleaving domain is fused to a nucleic acid binding polypeptide comprising for example a zinc finger as
10 described herein. Moreover, the invention provides therapeutic agents and methods of therapy involving use of nucleic acid binding polypeptides as described herein. In particular, the invention provides the use of polypeptide fusions comprising an integrase, such as a viral integrase, and a nucleic acid binding polypeptides according to the invention to target nucleic acid sequences *in vivo* (Bushman, 1994 *PNAS USA*
15 91:9233-9237). In gene therapy applications, the method may be applied to the delivery of functional genes into defective genes, or the delivery of nonsense nucleic acid in order to disrupt undesired nucleic acid. Alternatively, genes may be delivered to known, repetitive stretches of nucleic acid, such as centromeres, together with an activating sequence such as an LCR. This represents a route to the safe and predictable
20 incorporation of nucleic acid into the genome.

In conventional therapeutic applications, nucleic acid binding polypeptides according to the invention may be used to specifically knock out cell having mutant vital proteins. For example, if cells with mutant *ras* are targeted, they will be destroyed because *ras* is essential to cellular survival. Alternatively, the action of transcription
25 factors may be modulated, preferably reduced, by administering to the cell agents which bind to the binding site specific for the transcription factor. For example, the activity of HIV *tat* may be reduced by binding proteins specific for HIV TAR. Moreover, binding proteins according to the invention may be coupled to toxic molecules, such as nucleases, which are capable of causing irreversible nucleic acid
30 damage and cell death. Such agents are capable of selectively destroying cells which

comprise a mutation in their endogenous nucleic acid. Nucleic acid binding polypeptides and derivatives thereof as set forth above may also be applied to the treatment of infections and the like in the form of organism-specific antibiotic or antiviral drugs. In such applications, the binding proteins may be coupled to a nuclease
5 or other nuclear toxin and targeted specifically to the nucleic acids of microorganisms.

Poly-zinc finger peptides, with their ability to bind with high affinity to long (≥ 18 bp) DNA target sequences, are likely to be used more and more in the search for gene therapy treatments and applications such as transgenic plants / animals. However, for such applications to be effective and safe it is crucial that high affinity zinc finger
10 peptides are also highly specific. This is of particular importance given the extremely slow off rates observed for extended zinc finger arrays (Kim, J-S. & Pabo, C. O. (1998) *Proc. Natl. Acad. Sci. USA* **95**, 2812-2817). The zinc fingers disclosed in this document better satisfy both these requirements. We have achieved this by creating a design of six-finger peptides, which not only gives a slightly higher affinity than a
15 comparable 2x3F peptide, but more importantly, with far greater specificity for its full-length target. The two-finger units employed also allow greater flexibility in the selection of target sites by allowing one or two gaps of non-bound DNA, and reduce the library size required to select specific binding domains by techniques such as phage display. 3x2F peptides will greatly enhance the application of zinc finger arrays
20 for the *in vivo* control of gene expression.

Proteins and polypeptides suitable for treatment using the nucleic acid binding proteins of our invention include those involved in diseases such as cardiovascular, inflammatory, metabolic, infectious (viral, bacteria, fungul, etc), genetic, neurological, rheumatological, dermatological, and musculoskeletal diseases. In particular, the
25 invention provides nucleic acid binding proteins suitable for the treatment of diseases, syndromes and conditions such as hypertrophic cardiomyopathy, bacterial endocarditis, agyria, amyotrophic lateral sclerosis, tetralogy of fallot, myocarditis, anemia, brachial plexus, neuropathies, hemorrhoids, congenital heart defects, alopecia areata, sickle cell anemia, mitral valve prolapse, autonomic nervous system diseases,
30 alzheimer disease, angina pectoris, rectal diseases, arrhythmogenic right, ventricular

dysplasia, acne rosacea, amblyopia, ankylosing spondylitis, atrial fibrillation, cardiac tamponade, acquired immunodeficiency syndrome, amyloidosis, autism, brain neoplasms, central nervous system diseases, color vision defects, arteriosclerosis, breast diseases, central nervous system infections, colorectal neoplasms, arthritis, behcet's syndrome, breast neoplasms, cerebral palsy, common-cold, asthma, bipolar disorder, burns, cervix neoplasms, communication disorders, atherosclerosis, candidiasis, charcot-marie disease, crohn disease, attention deficit disorder, brain injuries, cataract, ulcerative colitis, cumulative trauma disorders, cystic fibrosis, developmental disabilities, eating disorders, erysipelas, fibromyalgia, decubitus ulcer, diabetes, emphysema, escherichia coli infections, folliculitis, deglutition disorders, diabetic foot, encephalitis, esophageal diseases, food hypersensitivity, dementia, down syndrome, japanese encephalitis, eye neoplasms, dengue, dyslexia, endometriosis, fabry's disease, gastroenteritis, depression, dystonia, chronic fatigue syndrome, gastroesophageal reflux, gaucher's disease, hematologic diseases, hirschsprung disease, hydrocephalus, hyperthyroidism, gingivitis, hemophilia, histiocytosis, hyperhidrosis, hypoglycemia, glaucoma, hepatitis, hiv infections, hyperoxaluria, hypothyroidism, glycogen storage disease, hepatolenticular degeneration, hodgkin disease, hypersensitivity, immunologic deficiency syndromes, hernia, holt-oram syndrome, hypertension, impotence, congestive heart failure, herpes genitalis, huntington's disease, pulmonary hypertension, incontinence, infertility, leukemia, systemic lupus erythematosus, maduromycosis, mental retardation, inflammation, liver neoplasms, lyme disease, malaria, inborn errors of metabolism, inflammatory bowel diseases, long qt syndrome, lymphangiomyomatosis, measles, migraine, influenza, low back pain, lymphedema, melanoma, mouth abnormalities, obstructive lung diseases, lymphoma, meningitis, mucopolysaccharidoses, leprosy, lung neoplasms, macular degeneration, menopause, multiple sclerosis, muscular dystrophy, myofascial pain syndromes, osteoarthritis, pancreatic neoplasms, peptic ulcer, myasthenia gravis, nausea, osteoporosis, panic disorder, myeloma, acoustic neuroma, otitis media, paraplegia, phenylketonuria, myeloproliferative disorders, nystagmus, ovarian neoplasms, parkinson disease, pheochromocytoma, myocardial diseases, opportunistic infections, pain, pars planitis, phobic disorders, myocardial infarction, hereditary optic atrophy, pancreatic diseases, pediculosis, plague, poison ivy dermatitis, prion diseases, reflex

- sympathetic dystrophy, schizophrenia, shyness, poliomyelitis, prostatic diseases, respiratory tract diseases, scleroderma, sjogren's syndrome, polymyalgia rheumatica, prostatic neoplasms, restless legs, scoliosis, skin diseases, postpoliomyelitis syndrome, psoriasis, retinal diseases, scurvy, skin neoplasms, precancerous conditions, rabies,
- 5 retinoblastoma, sex disorders, sleep disorders, pregnancy, sarcoidosis, sexually transmitted diseases, spasmodic torticollis, spinal cord injuries, testicular neoplasms, trichotillomania, urinary tract, infections, spinal dystaphism, substance-related disorders, thalassemia, trigeminal neuralgia, urogenital diseases, spinocerebellar degeneration, sudden infant death, thrombosis, tuberculosis, vascular diseases,
- 10 strabismus, tinnitus, tuberous sclerosis, post-traumatic stress disorders, syringomyelia, tourette syndrome, turner's syndrome, vision disorders, psychological stress, temporomandibular joint dysfunction syndrome, trachoma, urinary incontinence, von willebrand's disease, renal osteodystrophy, bacterial infections, digestive system neoplasms, bone neoplasms, vulvar diseases, ectopic pregnancy, tick-borne diseases,
- 15 marfan syndrome, aging, williams syndrome, angiogenesis factor, urticaria, sepsis, malabsorption syndromes, wounds and injuries, cerebrovascular accident, multiple chemical sensitivity, dizziness, hydronephrosis, yellow fever, neurogenic arthropathy, hepatocellular carcinoma, pleomorphic adenoma, vater's ampulla, meckel's diverticulum, keratoconus skin, warts, sick building syndrome, urologic diseases,
- 20 ischemic optic neuropathy, common bile duct calculi, otorhinolaryngologic diseases, superior vena cava syndrome, sinusitis, radius fractures, osteitis deformans, trophoblastic neoplasms, chondrosarcoma, carotid stenosis, varicose veins, creutzfeldt-jakob syndrome, gallbladder diseases, replacement of joint, vitiligo, nose diseases, environmental illness, megacolon, pneumonia, vestibular diseases, cryptococcosis,
- 25 herpes zoster, fallopian tube neoplasms, infection, arrhythmia, glucose intolerance, neuroendocrine tumors, scabies, alcoholic hepatitis, parasitic diseases, salpingitis, cryptococcal meningitis, intracranial aneurysm, calculi, pigmented nevus, rectal neoplasms, mycoses, hemangioma, colonic neoplasms, hypervitaminosis a, nephrocalcinosis, kidney neoplasms, vitamins, carcinoid tumor, celiac disease,
- 30 pituitary diseases, brain death, biliary tract diseases, prostatitis, iatrogenic disease, gastrointestinal hemorrhage, adenocarcinoma, toxic megacolon, amputees, seborrheic keratosis, osteomyelitis, barrett esophagus, hemorrhage, stomach neoplasms,

chickenpox, cholecystitis, chondroma, bacterial infections and mycoses, parathyroid neoplasms, spermatic cord torsion, adenoma, lichen planus, anal gland neoplasms, lipoma, tinea pedis, alcoholic liver diseases, neurofibromatoses, lymphatic diseases, elder abuse, eczema, diverticulitis, carcinoma, pancreatitis, amebiasis, pyelonephritis, and infectious mononucleosis, etc.

PHARMACEUTICAL COMPOSITIONS

The invention likewise relates to pharmaceutical preparations which contain the compounds according to the invention or pharmaceutically acceptable salts thereof as active ingredients, and to processes for their preparation. The pharmaceutical preparations according to the invention which contain the compound according to the invention or pharmaceutically acceptable salts thereof are those for enteral, such as oral, furthermore rectal, and parenteral administration to for example warm-blooded animal(s), the pharmacological active ingredient being present on its own or together with a pharmaceutically acceptable carrier. The dose of the active ingredient depends on the species, age and the individual condition and also on the manner of administration. For example, in the normal case, an approximate daily dose of about 10 mg to about 250 mg is to be estimated in the case of oral administration for a human patient weighing approximately 75 kg.

The novel pharmaceutical preparations contain, for example, from about 10 % to about 80%, preferably from about 20 % to about 60 %, of the active ingredient. Pharmaceutical preparations according to the invention for enteral or parenteral administration are, for example, those in unit dose forms, such as sugar-coated tablets, tablets, capsules or suppositories, and ampoules. These are prepared in a manner known in the art, for example by means of conventional mixing, granulating, sugar-coating, dissolving or lyophilising processes. Thus, pharmaceutical preparations for oral use can be obtained by combining the active ingredient with solid carriers, if desired granulating a mixture obtained, and processing the mixture or granules, if desired or necessary, after addition of suitable excipients to give tablets or sugar-coated tablet cores.

Suitable carriers are, in particular, fillers, such as sugars, for example lactose, sucrose, mannitol or sorbitol, cellulose preparations and/or calcium phosphates, for example tricalcium phosphate or calcium hydrogen phosphate, furthermore binders, such as starch paste, using, for example, corn, wheat, rice or potato starch, gelatin, 5 tragacanth, methylcellulose and/or polyvinylpyrrolidone, if desired, disintegrants, such as the abovementioned starches, furthermore carboxymethyl starch, crosslinked polyvinylpyrrolidone, agar, alginic acid or a salt thereof, such as sodium alginate; auxiliaries are primarily glidants, flow-regulators and lubricants, for example silicic acid, talc, stearic acid or salts thereof, such as magnesium or calcium stearate, and/or 10 polyethylene glycol. Sugar-coated tablet cores are provided with suitable coatings which, if desired, are resistant to gastric juice, using, inter alia, concentrated sugar solutions which, if desired, contain gum arabic, talc, polyvinylpyrrolidone, polyethylene glycol and/or titanium dioxide, coating solutions in suitable organic solvents or solvent mixtures or, for the preparation of gastric juice-resistant coatings, 15 solutions of suitable cellulose preparations, such as acetylcellulose phthalate or hydroxypropylmethylcellulose phthalate. Colorants or pigments, for example to identify or to indicate different doses of active ingredient, may be added to the tablets or sugar-coated tablet coatings.

Other orally utilisable pharmaceutical preparations are hard gelatin capsules, 20 and also soft closed capsules made of gelatin and a plasticiser, such as glycerol or sorbitol. The hard gelatin capsules may contain the active ingredient in the form of granules, for example in a mixture with fillers, such as lactose, binders, such as starches, and/or lubricants, such as talc or magnesium stearate, and, if desired, stabilisers. In soft capsules, the active ingredient is preferably dissolved or suspended 25 in suitable liquids, such as fatty oils, paraffin oil or liquid polyethylene glycols, it also being possible to add stabilisers.

Suitable rectally utilisable pharmaceutical preparations are, for example, suppositories, which consist of a combination of the active ingredient with a suppository base. Suitable suppository bases are, for example, natural or synthetic 30 triglycerides, paraffin hydrocarbons, polyethylene glycols or higher alkanols.

Furthermore, gelatin rectal capsules which contain a combination of the active ingredient with a base substance may also be used. Suitable base substances are, for example, liquid triglycerides, polyethylene glycols or paraffin hydrocarbons.

Suitable preparations for parenteral administration are primarily aqueous solutions of an active ingredient in water-soluble form, for example a water-soluble salt, and furthermore suspensions of the active ingredient, such as appropriate oily injection suspensions, using suitable lipophilic solvents or vehicles, such as fatty oils, for example sesame oil, or synthetic fatty acid esters, for example ethyl oleate or triglycerides, or aqueous injection suspensions which contain viscosity-increasing substances, for example sodium carboxymethylcellulose, sorbitol and/or dextran, and, if necessary, also stabilisers.

TWO FINGER MODULE LIBRARIES

The present invention includes a method of constructing multi-finger zinc finger proteins which are based on a construction unit of two fingers. The use of combinatorial libraries for generating two-zinc finger DNA binding domains is disclosed. We further describe a number of linkers that are suitable in constructing multifinger proteins and that are especially suitable for use with construction units of two fingers.

According to this aspect of the invention, combinatorial library systems may be used to generate two-finger construction units. Such libraries take advantage on a number of features of the libraries described in published patent applications WO 98/53057, WO 98/53058, WO 98/53059, and WO 98/53060 which are hereby incorporated by reference. In particular, the libraries are constructed in such a way as to enable the synergistic interaction between the two fingers which comprise the selected two-finger construction unit to be utilised.

We have determined that DNA-binding subunits comprising two-zinc finger domains may be engineered through the variety of approaches described herein, each

of which has distinct advantages for creating DNA-binding proteins. In each of the libraries detailed here, amino acid randomizations are made at various positions in the two zinc finger structures. Preferred randomizations are described here as well as in patent applications WO 96/06166, WO 98/53057, WO 98/53058, WO 98/53059, and
5 WO 98/53060. However, a more restricted number of randomizations may be utilized in library construction to facilitate the process of construction. The library construction methods described herein can be used in conjunction with a variety of selection methods including phage display and ribosome display as detailed in patent applications WO 97/53057 and WO 00/27878, both of which are incorporated herein
10 by reference.

In one approach, an isolated two finger library is constructed, which comprises amino acids known to contribute to DNA-binding affinity and specificity. Since the library does not contain a DNA-binding "anchor", the register of the interaction is not strictly fixed, so this library may suitably be used for applications where either (i) the
15 precise register of interaction is not critical for subsequent applications, or (ii) very short DNA targets [6-7 bp] are used in the selection procedure, thereby fixing the interaction more precisely.

It is highly desirable to engineer 2-finger domains whose register of interaction is precisely fixed, and which can be targeted to any DNA sequence. We have shown
20 that this can be achieved by employing "GCG" anchors (although any other anchor sequence can be employed) and two extensively-randomised zinc fingers. The libraries are designed to take into account synergistic effects between zinc fingers, by modifying cross-strand contacts from position 2. Consequently, position 2 of F2 is modified to Ser or Ala so as to interact universally with either the 7'C in the "GCG"
25 anchor, or any base (7'N) in the final target site sequence. Similarly, position 2 of F3 is modified to Ser or Ala so as not to interfere with the selection of bases 4'X or 4'X. As before, after selecting against particular DNA target sites, the genes for the appropriate 2-finger domains may be easily recovered by PCR.

In a further approach, two previously constructed libraries (Lib12 and Lib23, as described in WO 98/53057) are readily adapted to provide a resource of 2-finger subunits. These two libraries have been extensively characterised and used for the selection of zinc finger modules of 1.5 fingers, each of which is then recombined to generate a 3-finger module (see WO98/50357). We now show that these libraries can be used to select two finger units that bind DNA sites of the form 5'-GXX XXX-3' or 5'-XXX XXG-3' (where X is any base). After selecting against particular DNA target sites, the genes for the appropriate 2-finger domains may be easily recovered by PCR. Because of the design of the libraries, the "GCGG" or "GGCG" anchors serve to fix the register of DNA-protein interaction very precisely. Despite the fact that one base must be fixed as "G" in each target site, this still allows 2048 of all the 4096 ($=4^6$) possible 6-base 2-finger recognition sites to be targeted.

The general principle is demonstrated below.

15	Library	Binding Site (5'-3')		
		F3	F2	F1
	LIB12	GCG	GXX	XXX
20	LIB23	XXX	XXG	GCG

Therefore, LIB12 may be used to select a novel 2-finger unit that binds a 6 bp site with a 5' guanine. Similarly, LIB23 can be used to select a novel 2-finger unit that binds a 6 bp site with a 3' guanine.

25 Accordingly, we have recognized that the concept of selection of two-finger construction units need not require full randomization of both zinc fingers as libraries can be generated which providing for the fixing of one (or more) of the base contacting positions and selection against a DNA sequence that incorporates the corresponding nucleotide at the pre-determined base contacting position. Libraries may, for example, be constructed from zinc finger proteins in which two of the nucleotides of either target triplet are fixed. Using Zif268 as the backbone this would, for example, allow

selection of two finger modules which target the sequence 5'-GGNNNN-3' or 5'-NNNNGG-3'. Using other backbone zinc fingers, the fixed nucleotides may be other nucleotides.

In an extension of this concept, it will be appreciated that Lib12 and Lib23 can be used to select 2-finger domains which bind the sequences GCGGXX or XXGGCG respectively.

Further advantages offered by 2-finger domains include the following: (a) the 2-finger domains are independent so no problems are encountered when fusing separately selected units; (b) no further rounds of selection are required after selecting individual 2-finger domains; (c) 3x2F peptides are more specific than 2x3F peptides; (d) 3x2F peptides allow two 1 bp gaps to be accommodated within the target sequence; (e) with minor modifications to the libraries any 6 bp sequence can be targeted in one go; (f) complete binding site signatures may be possible for entire 2-finger units by DNA micro-array ELISA. Thus, as indicated in (d) above, 3x2F peptides allow two 1 bp gaps to be accommodated within the target sequence, indeed 2-finger units bind with optimal efficiency when within 1 bp of each other.

The invention is further described, for the purposes of illustration only, in the following examples.

EXAMPLES

Example 1: Constructs, Targets and Nomenclature

In order to combine the benefits of tight binding to an extended DNA sequence, coupled with the flexibility to skip bases in the DNA target site, we designed a series of six fingered chimeric zinc finger proteins derived from wild type ZIF fused to a GAC-clone. Each construct comprises three pairs of zinc fingers separated by extended, flexible linker peptides. These are termed "3x2F peptides".

One such flexible linker construct comprises the fingers of the wt ZIF and GAC with zinc finger pairs separated by $-GG^E/QKP-$ and is termed 3x2F ZGS (Figure 3). This peptide targets the contiguous DNA binding sequence, bsC (Table 1), which comprises the wt ZIF and GAC-clone binding sites. To allow some variation in the binding sites targeted by the 3x2F protein, finger pairs are also separated by $-GGSG^E/QKP-$, or $-GGSGGSG^E/QKP-$ linker sequences to create the 3x2F ZGL and 3x2F ZGXL constructs respectively (Figures 4 and 5). These peptides are targeted against the contiguous ZIF-GAC binding site (bsC), and against the binding sites bsD and bsE (Table 1), which contain 1 or 2 bps, respectively, between the recognition sequences for the zinc finger pairs. Similar constructs are also synthesised in which two-finger units are separated by linkers containing either glycine or Gly-Gly-Ser insertions. These constructs are termed 3x2F ZGSL and 3x2F ZGLS (Figures 6 and 7) and are targeted against the appropriate binding sites, bsF and bsG (Table 1).

Constructs are also made comprising structured linkers. One such construct comprises the first four fingers of TFIIIA (including the F4-F5 linker peptide) joined to the N-terminus of the three-finger ZIF peptide. The resultant seven-finger peptide is denoted TF(F1-4)-ZIF (Example 15 and Figures 13 and 15), and is targeted to non-contiguous binding sites containing the TFIIIA F1-3 and wt ZIF sites separated by 5 to 10 bps of DNA (Table 2). The second construct is created by substituting the first three fingers of TFIIIA in the above fusion peptide with the three-finger GAC-clone, and is denoted GAC-F4-ZIF (Example 16 and Figures 14 and 16). This peptide is targeted against the non-contiguous binding sites (Table 3), which comprise the GAC-clone and wt ZIF recognition sites separated by 6 to 11 bps of DNA. A third structured linker construct is ZIF-ZnF-GAC which consists of the three finger peptide of ZIF linked to a three fingered GAC-clone using a "neutral" finger linker, i.e., a wild type ZIF268 finger 2 with the amino acids at positions -1,2,3 and 6 replaced with serine residues.

Further constructs are also made. ZIF-F4-GAC comprises finger 4 of TFIIIA inserted between Zif268 and the mutant Zif268 clone GAC (which is a phage selected variant of Zif268 capable of binding GCG GAC GCG). The linkers found naturally in TFIIIA between finger 3 and finger 4 (-NIKICV-) and between finger 4 and finger 5 (-

- TQQLP-) are retained in both the above peptides. ZIF-F4mut-GAC is identical to ZIF-F4-GAC, except that the linkers flanking finger 4 of TFIIIA are replaced by canonical linkers having the sequence GERP. ZIF-mutZnF-GAC is identical to ZIF-ZnF-GAC, except that the TFIIIA finger 4 flanking sequences comprise -NIKICV- and -TQQLP-.
- 5 TF(1-3)-flex-ZIF and ZIF-flex-GAC contain the 20 amino acid sequence: -TG(GSG)₅ERP- between their respective three-finger domains.

Example 2: Construction of 3x2F ZGS Zinc Finger Construct

- The 3x2F ZGS zinc finger construct is created by linking the third finger of wild-type ZIF to the first finger of the GAC-clone using the peptide sequence GERP.
- 10 To divide the new peptides into three pairs of fingers, one glycine residue is inserted into the peptide linker between fingers 2 and 3 of wild type ZIF and between fingers 1 and 2 of the GAC-clone. The amino acid and nucleotide sequences of the 3x2F ZGS construct are shown in Figure 3.

- The construction of 3x2F ZGS is described with reference to Figures 1 and 3.
- 15 As shown in Figure 1, the 3x2F ZGS construct is made by mutagenic PCR of wild type ZIF and GAC-clone templates. ZIF and GAC-clone templates are as described in Choo & Klug (1994), *Proc. Natl. Acad. Sci USA* 91,11163-11167. Four pairs of oligonucleotide primers, A + a, B + b, C + c and D + d are used. As indicated in Figure 1, primers A, a, B and b are used to amplify and mutagenise wild type ZIF sequence,
- 20 while primers C, c, D and d are used to amplify the GAC-clone. The sequences of primers A and d comprise restriction sites for *NdeI* and *NotI* respectively, while primers C and b comprise *EagI* recognition sites. Primers B and D are mutagenic oligonucleotides, whose sequences comprise linker sequences from wild type ZIF (primer B) and GAC (primer D) but with additional nucleotide sequence coding for
- 25 additional amino acid residues. These linker sequences are chosen from the linker between finger 2 and finger 3 of wild type ZIF (primers a and B) and the linker between finger 1 and finger 2 of the GAC clone (primers c and D). For example, in the case of 3x2F ZGS, primers B and D each include an additional GGC triplet to code for glycine.

To construct the 3x2F ZGS clone, wild type ZIF sequence is amplified by means of primers A, a, B and b, while GAC-clone sequence is amplified by means of primers C, c, D and d. The respective amplification products are then subjected to overlap PCR, with a template fill-in step. Finally, each of the products is amplified
 5 with end primers A + b and C + d. The amplification products are then digested with *EagI*, and ligated at that site. The full length product comprising sequence encoding the 6 finger protein is then digested with *NotI* and *NdeI*, and ligated into *NotI/NdeI* digested pCITE-4b vector (Amersham International Plc). pCITE4b is a eukaryotic expression vector containing a T7 transcription promoter and an internal eukaryotic
 10 ribosome translation entry site for protein expression. Plasmids containing the zinc-finger constructs are propagated in *E. coli* XL1-Blue (Stratagene) cells.

The sequences of oligonucleotide primer sequences A, a, B, b, C, c, D and d for construction of 3x2F ZGS are shown below, in which restriction sites used in cloning and inserted glycine codons are shown in bold, while annealing sequences for PCR are
 15 underlined:

Primer A (SEQ ID NO: 1):

5' CAG CCG GCC ^{Nde I} CAT ATG CGT CTA GAC GCC GCC ^{START} ATG GCA GAA CGC CCG TAT GCT TG 3'

Primer a (SEQ ID NO: 2):

5' CTG TGT GGG TGC GGA TGT GGG T 3'

Primer B (SEQ ID NO: 3):

5' ACC CAC ATC CGC ACC CAC ACA GGT ^{Gly} GGC GAG AAG CCT TTT GCC 3'

Primer b (SEQ ID NO: 4):

5' GCA AGC ATA ^{Eag I} CGG CCG TTC ACC GGT ATG GAT TTT GGT ATG CCT CTT GCG T 3'

Primer C (SEQ ID NO: 5):

5' ATG GCA ^{Eag I} GAA CGG CCG TAT GCT TGC CC 3'

Primer c (SEQ ID NO: 6):

5' GTG TGG ATG CCG ATA TGG CCG GT 3'

Primer D (SEQ ID NO: 7):

5' CCC GCC ATA TCC GCA TCC ACA CAG ^{Gly} GTG GCC AGA AGC CCT TCC AG 3'

Primer d (SEQ ID NO: 8):

Not I STOP
5' TCA TTC AAG TGC GGC CGC TTA GGA ATT CCG GGC
CGC GTC CTT CTG TCT TAA ATG GAT TTT GG 3'

Example 3: Construction of the ZIF-GAC Fusion Construct

The control construct ZIF-GAC is created by joining the third finger of ZIF to the first finger of the GAC-clone using the peptide sequence described by Kim and Pabo (1998, *Proc. Natl. Acad. Sci. USA* 95, 2812-2817), -LRQKDGERP-. This linker is designed to have compatible ends with the adjacent zinc finger sequences. A modification of the method as described above for Example 2 is used. Thus, primers A and b (primer b having the sequence shown below) is used to amplify wild type ZIF, while primers C and d are used to amplify the GAC clone, and the two amplified sequences joined together. The amino acid and nucleotide sequence of the ZIF-GAC fusion construct is shown in Figure 2. The oligonucleotide primer sequences A; C and d as shown in Example 2 are used for constructing ZIF-GAC, except that primer b has the following sequence:

Primer b (SEQ ID NO: 9):

20 *Eag* I Gly
5' GCA AGC ATA CGG CCG TTC GCC GTC CTT CTG TCT TAA ATG GAT TTT GG 3'

Example 4: Construction of 3x2F ZGL Zinc Finger Construct

The 3x2F ZGL construct is created using the same method as described above for Example 2, except that amino acid residues GGS are inserted into the linker sequence between fingers 2 and 3 of wild type ZIF and into the linker sequence between fingers 1 and 2 of the GAC-clone. The amino acid and nucleotide sequence of 3x2F ZGL is shown in Figure 4. The oligonucleotide primer sequences used for constructing 3x2F ZGL are the same as for 3x2F ZGS (Example 2), except for the following:

Primer B (SEQ ID NO: 10):

Gly Gly Ser

5' ACC CAC ATC CGC ACC CAC ACA GGC GGT TCT GGC GAG AAG CCT TTT GCC 3'

5 Primer D (SEQ ID NO: 11):

Gly Gly Ser

5' CCC GCC ATA TCC GCA TCC ACA CAG GCG GTT CTG GCC AGA AGC CCT TCC AG 3'

Example 5: Construction of 3x2F ZGXL Zinc Finger Construct

- 10 The 3x2F ZGXL construct is created using the same method as described above for Example 2, except that amino acid residues GGS are inserted into the linker sequence between fingers 2 and 3 of wild type ZIF and into the linker sequence between fingers 1 and 2 of the GAC-clone. The amino acid and nucleotide sequence of 3x2F ZGXL is shown in Figure 5. The oligonucleotide primer sequences used for
- 15 constructing 3x2F ZGXL are the same as for 3x2F ZGS (Example 2), except for the following:

Primer B (SEQ ID NO: 12):

Gly Gly Ser Gly Gly Ser

20 5' ACC CAC ATC CGC ACC CAC ACA GGC GGT TCT GGC GGT TCT GGC GAG AAG CCT TTT
GCC 3'

Primer D (SEQ ID NO: 13):

Gly Gly Ser Gly Gly Ser

25 5' CCC GCC ATA TCC GCA TCC ACA CAG GCG GTT CTG GCG GTT CTG GCC AGA AGC CCT
TCC AG 3'

Example 6: Construction of 3x2F ZGSL Zinc Finger Construct

- 30 The 3x2F ZGSL construct is created using the same method as described above for Example 2, except that a single glycine residue is inserted into the linker sequence between fingers 2 and 3 of wild type ZIF, and amino acid residues GGS are inserted into the linker sequence between fingers 1 and 2 of the GAC-clone. The amino acid and nucleotide sequence of 3x2F ZGSL is shown in Figure 6. The oligonucleotide

primer sequences used for constructing 3x2F ZGSL are the same as for 3x2F ZGS (Example 2), except for the following:

Primer D (SEQ ID NO: 11):

5' CCC GCC ATA TCC GCA TCC ACA CAG ^{Gly Gly Ser} GCG GTT CTG GCC AGA AGC CCT TCC AG 3'

Example 7: Construction of 3x2F ZGLS Zinc Finger Construct

The 3x2F ZGLS construct is created using the same method as described above for Example 2, except that amino acid residues GGS are inserted into the linker sequence between fingers 2 and 3 of wild type ZIF, and a single glycine residue is inserted into the linker sequence between fingers 1 and 2 of the GAC-clone. The amino acid and nucleotide sequence of 3x2F ZGLS is shown in Figure 7. The oligonucleotide primer sequences used for constructing 3x2F ZGLS are the same as for 3x2F ZGS (Example 2), except for the following:

Primer B (SEQ ID NO: 10):

5' ACC CAC ATC CGC ACC CAC ACA GGC ^{Gly Gly Ser} GGT TCT GGC GAG AAG CCT TTT GCC 3'

20 Example 8: Protein Expression

The zinc-finger constructs are expressed *in vitro* by coupled transcription and translation in the TNT Quick Coupled Transcription/Translation System (Promega) using the manufacturer's instructions, except that the medium is supplemented with ZnCl₂ to 500 µM. To judge relative protein expression levels, translation products are labelled with ³⁵S-met and visualised by autoradiography, following SDS-PAGE.

Example 9: Gel Shift Assays

All constructs are assayed using ³²P end-labelled synthetic oligonucleotide duplexes containing the required binding site sequences. The coding strand sequences

of the binding sites used in gel shift experiments with peptides containing flexible linkers are shown below in Table 1. Table 2 shows the coding strand sequences of the binding sites used in gel shift experiments with peptides containing structured linkers.

- 5 DNA binding reactions contain the appropriate zinc-finger peptide, binding site and 1 µg competitor DNA (poly dI-dC) in a total volume of 10 µl, which contains: 20 mM Bis-tris propane (pH 7.0), 100 mM NaCl, 5 mM MgCl₂, 50 µM ZnCl₂, 5 mM DTT, 0.1 mg/ml BSA, 0.1% Nonidet P40. Incubations are performed at room temperature for 1 hour.

Name	Sequence	Putative target for construct	SEQ ID:
bsA	GCG TGG GCG	Wild type ZIF/3x1F Zif	14
bsB	GCG GAC GCG	GAC-clone (wild-type binding site sequences for fingers 1 and 3, middle finger binds GAC)	15
bsC	GCG GAC GCG GCG TGG GCG	ZIF-GAC and 3x2F ZGS (contiguous 18 bp site comprising wt ZIF and GAC-clone sites)	16
bsD	GCG GAC T GCG GCG T TGG GCG	3x2F ZGL (2-finger / 6 bp sites separated by 1 bp)	17
bsE	GCG GAC TC GCG GCG TC TGG GCG	3x2F ZGXL (2-finger / 6 bp sites separated by 2 bps)	18
bsF	GCG GAC T GCG GCG TGG GCG	3x2F ZGSL (1 bp gap between the binding sites for the first and second fingers of the GAC-clone)	19
bsG	GCG GAC GCG GCG T TGG GCG	3x2F ZGLS (1 bp gap between the binding sites for the second and third fingers of wtZIF)	20

- 10 **Table 1.** The binding site sequences contained within the oligonucleotides used in gel shift experiments with peptides containing flexible linkers.

Name	Sequence	Notes	SEQ ID:
bsA1	GCGTGGGCGTACCTGGATGGGAGAC	ZIF and TFIIIA (F1-3) binding sites separated	39

		by 5 nucleotides	
bsB1	GCGTGGGCGGTACCTGGATGGGAGAC	ZIF and TFIIA (F1-3) binding sites separated by 6 nucleotides	40
bsC1	GCGTGGGCGAGTACCTGGATGGGAGAC	ZIF and TFIIA (F1-3) binding sites separated by 7 nucleotides	41
bsD1	GCGTGGGCGTAGTACCTGGATGGGAGAC	ZIF and TFIIA (F1-3) binding sites separated by 8 nucleotides	42
bsE1	GCGTGGGCGTTAGTACCTGGATGGGAGAC	ZIF and TFIIA (F1-3) binding sites separated by 9 nucleotides	43
bsF1	GCGTGGGCGTTAGTACCTGGATGGGAGAC	ZIF and TFIIA (F1-3) binding sites separated by 10 nucleotides	44
bsG1	GCGTGGGCGCTTGACGGATGGGAGAC	ZIF and TFIIA (F1-3) binding sites separated by 6 nucleotides	45
bsH1	GCGTGGGCGAAAAAGGATGGGAGAC	ZIF and TFIIA (F1-3) binding sites separated by 6 nucleotides	46

Table 2. The binding site sequences contained within the oligonucleotides used in gel shift experiments with the TFIIA (F1-4)-ZIF peptide. The binding site sequences of TFIIA F1-3 and wild-type ZIF (bold) are separated by between 5 and 10 bps of DNA. The DNA sequence used to separate the binding sites is based on the sequence spanned by TFIIA-finger 4 in the Internal Control Region of the 5S rRNA gene – TFIIA's natural binding site. To investigate any possible sequence preference for the region spanned by TFIIA-finger 4, oligonucleotides containing an altered sequence (bsG1), or 6 adenine residues (bsH1) are designed and tested in bandshifts.

10

Name	Sequence	Notes	SEQ ID
bsA2	GCGTGGGCGTACCTGGCGGACGCG	ZIF and GAC-clone binding sites separated by 6 nucleotides	47
bsB2	GCGTGGGCGGTACCTGGCGGACGCG	ZIF and GAC-clone binding sites separated by 7 nucleotides	48
bsC2	GCGTGGGCGAGTACCTGGCGGACGCG	ZIF and GAC-clone binding sites separated by 8 nucleotides	49
bsD2	GCGTGGGCGTAGTACCTGGCGGACGCG	ZIF and GAC-clone binding sites separated by 9 nucleotides	50

bsE2	GCCTGGGCGTTAGTACCTGGCGGACGCG	ZIF and GAC-clone binding sites separated by 10 nucleotides	51
bsF2	GCCTGGGCGTTAGTACCTGGCGGACGCG	ZIF and GAC-clone binding sites separated by 11 nucleotides	52

Table 3. The binding site sequences contained within the oligonucleotides used in gel shift experiments with the GAC-F4-ZIF peptide. The binding site sequences of the GAC-clone and wild-type ZIF (bold) are separated by between 6 and 11 bps of DNA. The DNA sequence spanned in each case is based on the sequence spanned by TFIIIA-finger 4 in the ICR of the 5S rRNA gene, as described above in Figure 2.

Relative dissociation constants are determined by creating 5-fold serial dilutions of the required peptide and incubating with the appropriate binding site at a constant concentration, which is in general between 0.1 and 0.2 nM. The concentration of protein at which 50% of the binding site is bound is compared for each peptide, with either the full length or part-binding site sequences, to assess the difference in binding affinity. In cases where a non-total bandshift appears only in lanes containing the lowest concentration of peptide, it is likely that the amount of shift is limited by protein concentration rather than by affinity. Therefore, the relative difference in affinity is likely to be greater than that observed and shown.

Example 9A. Active Peptide Concentration

To determine the concentration of zinc finger peptide produced in the *in vitro* expression system, crude protein samples are used in gel-shift assays against a dilution series of the appropriate binding site. Binding site concentration is always well above the K_d of the peptide, but ranged from a higher concentration than the peptide (80 mM), at which all available peptide binds DNA, to a lower concentration (3-5 mM), at which all DNA is bound. Controls are carried out to ensure that binding sites are not shifted by the *in vitro* extract in the absence of zinc finger peptide. The reaction mixtures are then separated on a 7% native polyacrylamide gel. Radioactive signals are quantitated by PhosphorImager analysis to determine the amount of shifted binding site, and hence, the concentration of active zinc finger peptide.

Example 9B. Binding Affinity and Specificity

Dissociation constants are determined in parallel to the calculation of active peptide concentration. Serial 3, 4 or 5-fold dilutions of crude peptide are made and incubated with radiolabelled binding site (0.1 pM – 500 pM depending on the peptide),
5 as above. Samples are run on 7% native polyacrylamide gels and the radioactive signals quantitated by PhosphorImager analysis. The data is then analysed according to linear transformation of the binding equation and plotted in CA-Cricket Graph III (Computer Associates Inc. NY) to generate the apparent dissociation constants. The K_d values reported are the average of at least two separate studies.

10 Example 10: Binding Affinity of the Control Construct ZIF-GAC

In order to compare the binding affinities of the various constructs described here, the ZIF-GAC peptide is used as a control. This peptide may be thought of as a pair of three-finger peptides, and accordingly may be designated as 2x3F. The ZIF-GAC construct is tested for binding to the binding site bsC and to the ZIF binding site
15 alone (bsA). The results are shown in Figure 9A. Figure 9A show that the composite site bsC is bound 125-500 fold more tightly than the 9bp bsA site. This result is comparable to that observed when the experiment of Kim and Pabo (1998, *Proc. Natl. Acad. Sci. USA* 95, 2812-2817) is repeated using our methods of protein production and bandshift, ie testing the ZIF-NRE peptide for binding to its composite site versus
20 the ZIF wt site.

Example 11: Binding Affinities of Constructs 3x2F ZGS and 3x2F ZGL

The binding affinities of ZIF-GAC, 3x2F ZGS and 3x2F ZGL peptides for a contiguous 18 bp site (bsC) and the 9 bp ZIF binding site (bsA) alone are determined. Serial five-fold dilutions of peptide are made and incubated with 0.13 nM binding site.
25 Significantly, the results show that the 3x2F peptides bind the contiguous 18 bp site at least as tightly as the 2x3F ZIF-GAC peptide (Figures 9A and 9B). Moreover, the 3x2F peptides display greater selectivity for the 18 bp site over the 9 bp site, than does

the 2x3F peptide. The affinity of the 3x2F peptides for the 9bp half-site is reduced due to the extended linker sequence between fingers 2 and 3 of ZIF. The expression level of the 3x2F ZGL peptide is approximately half that of the ZIF-GAC and 3x2F ZGS peptides in this study, which accounts for its slightly weaker apparent affinity
5 (expression data not shown).

Example 12: Binding Affinities of Constructs 3x2F ZGS, 3x2F ZGL and 3x2F ZGXL

The next experiment is designed to determine whether 3x2F peptides can be used to bind non-contiguous sites with two separate regions of unbound DNA. The
10 constructs used in this study are 3x2F ZGS, 3x2F ZGL and 3x2F ZGXL, and are targeted to the sequences of bsC, bsD and bsE. These sequences can be described as comprising three sets of 6 bp sub-sites, which are either contiguous, separated by 1 bp or separated by 2 bps of unbound DNA.

As shown in Figure 9B, the results demonstrate that the 3x2F ZGS and 3x2F
15 ZGL peptides bind the contiguous 18bp site (bsC) equally tightly (taking into account the different protein expression levels). We also find that the 3x2F ZGL peptide can bind the non-contiguous site (bsD) as tightly as it does the contiguous 18bp site bsC (see Figures 9B and 10). However, the 3x2F ZGS peptide binds bsD over 125-fold more weakly than it does bsC (compare left hand panels of Figure 9B and Figure 10).
20 This is in accordance with the fact that the short, five amino acid synthetic linkers within 3x2F ZGS are unable to span 1 bp of DNA, and therefore the 3x2F ZGS peptide binds the bsD site through only one pair of fingers.

Figure 11 shows that the 3x2F ZGXL peptide can bind the non-contiguous site (bsD) as tightly as it does the contiguous 18bp site bsC. 3x2F ZGXL binds the non-
25 contiguous site bsD approximately as tightly as the 3x2F ZGS peptide binds the contiguous 18bp site, bsC. However, the 3x2F ZGXL peptide binds bsE (containing 2 base pair gaps between target subsites) approximately 500-fold less tightly than it does

bsC and bsD, as shown in Figure 11. This is presumably because it can only bind bsE through 2 fingers.

Example 13: Binding Affinities of Constructs 3x2F ZGSL and 3x2F ZGLS

As a continuation of the above experiment, 3x2F peptides are constructed with
5 different combinations of engineered linkers within a ZIF-GAC fusion peptide. In the construct 3x2F ZGSL the first two pairs of fingers are separated by a short ($-GG^E/QKP-$) linker and the second two pairs are separated by a longer ($-GGSG^E/QKP-$) linker (see Figure 6). In the construct 3x2F ZGLS the first two pairs of fingers are separated by a long ($-GGSG^E/QKP-$) linker and the second two pairs are
10 separated by a shorter ($-GG^E/QKP-$) linker (see Figure 7).

These two peptides are tested for binding to binding sites bsF, which has a 1 bp gap between the first two 6bp subsites, and bsG which has a 1 bp gap between the second two 6bp subsites (see Table 1). As expected, given the previous observations, the results demonstrate that the binding of arrays of zinc finger pairs can be tailored to
15 suit the length of gap between 6 bp binding subsites. Figure 12 shows the results of a gel shift experiment testing the binding of 3x2F ZGSL peptide to bsD, bsE and bsF, which is through 4, 2 and 6 fingers respectively. From the binding patterns it can be seen that the affinity of the 6-finger bound complex (3x2F ZGSL on bsF, right hand panel) is approximately 10-fold higher than the 4-finger bound complex (3x2F ZGSL on bsD, middle panel) and 125-500 fold stronger than the 2-finger bound complex
20 (3x2F ZGSL on bsE, left hand panel).

Similarly, 3x2F ZGLS peptide is tested for binding to bsD, bsE and bsG, which is through 4, 2 and 6 fingers respectively. It is found that the affinity of binding of 3x2F ZGLS is strongest for bsG, followed by bsD and lastly bsE, with relative
25 affinities similar to those obtained from 3x2F ZGSL above.

Example 13A: Binding Affinity of 3x2F ZGS and Zif-GAC

A preliminary experiment is conducted using the three-finger Zif268 peptide against its 9 bp binding site as a form of 'protocol calibration'. This gives a value for the K_d of Zif268 of 0.45 nM, which is within the range expected for this peptide.

- 5 To determine the binding specificity of different styles of six-finger peptides, the 3x2F ZGS and Zif-GAC peptides are first used in gel-shift experiments with the 9 bp Zif268 half-site, and a full 18 bp binding site (bsC, also termed "123456"). These results show that the 3x2F ZGS and 2x3F Zif-GAC peptides bind their full-length target site with similar affinities, of 0.6 and 1.4 pM respectively (Table 4 below).
- 10 However, their affinities for the Zif268 half-site are dramatically different. The 2x3F Zif-GAC peptide binds with an affinity of approximately 2.2 nM (which is within the range expected), but the 3x2F ZGS peptide binds with an affinity of about 110 nM. This affinity is so weak that it is difficult to quantify using this system. From these data it can be seen that the 3x2F peptide discriminates between the two sites over 100-
- 15 fold more strongly than the 2x3F peptide.

- To further study the specificity of the two constructs the 3x2F and 2x3F peptides are targeted against binding sites that have been mutated in the region normally bound by finger 4. These results show that the 3x2F ZGS peptide binds to the site with a 3 bp region mutated, 123///56, with an affinity of 890 pM. Meanwhile, it
- 20 binds to a site with this 3 bp region deleted, 12356, with an affinity of 22 nM (see Table 5 below). Its affinities for sites with 1 or 2 bp deletions are 270 pM and 630 pM respectively. Hence, the affinities of 3x2F ZGS for these mutant sequences are between 450 and 37,000-fold weaker than for the correct binding sequence. In contrast, the 2x3F Zif-GAC peptide binds 123///56, 123//56, and 123/56 with affinities
- 25 of 15, 14 and 14 pM respectively. This is just 10-fold weaker than that for its correct binding site. The 2x3F Zif-GAC peptide shows a further reduction in affinity for the 12356 binding site, but this sequence is still bound more than 60 times stronger than it is bound by 3x2F ZGS. The gel-shift data in Figure 25 demonstrates the relative binding affinities of the 2x3F Zif-GAC and 3x2F ZGS peptides for these binding sites.

All this data serves to emphasise the enhanced specificity of the 3x2F construct for sequences that resemble its correct target site. The gel-shift data of Figure 25 demonstrates the relative affinities of the 3x2F ZGS and 2x3F Zif-GAC peptides for the target sites.

5

Binding Site Name	Binding Site Sequence *	Apparent Kd (pM)	
		3x2F ZGS	2x3F Zif- GAC
bsA (ZIF)	GCG TGG GCG	1.1×10^3	2200
123456 (bsC)	GCG GAC GCG GCG TGG GCG	0.6	1.4
123///56 (bs4)	GCG GAC <u>ATC</u> GCG TGG GCG	890	15
123//56 (bs3)	GCG GAC <u>TC</u> GCG TGG GCG	270	14
123/56 (bs2)	GCG GAC <u>T</u> GCG TGG GCG	630	14
12356	GCG GAC GCG TGG GCG	2.2×10^4	360

Table 4. The binding site sequences used in gel-shift experiments with the 3x2F ZGS and 2x3F Zif-GAC peptides and the binding affinities obtained. * Binding site residues which are mutated (and subsequently deleted) are underlined.

Example 13B. Binding of Non-Contiguous Sequences

10 A second set of binding studies is conducted to demonstrate the ability of the 3x2F peptides to accommodate one or more regions of unbound DNA within their recognition sequence. First the 3x2F ZGS and ZGL peptides are titrated against 12/34/56 (three 6 bp subsites separated by 1 bp, which is represented by a single '/' in the binding site name) and 12//34//56 (three 6 bp subsites separated by 2 bps) binding
15 sites. The results in Table 5 show that the 3x2F ZGS peptide - which is designed to target only the contiguous 123456 site - is unable to accommodate either 1 bp or 2 bp gaps between the two-finger subsites. The 3x2F ZGL peptide, however, binds the 12/34/56 site with an affinity of approximately 5 pM, but is also unable to bind tightly to the site with 2 bp gaps. Next, the 3x2F ZGSL and 3x2F ZGLS peptides are targeted

- against the three non-contiguous sequences: 1234/56, 12/3456 and 12//34//56. These sites are bound by the 3x2F ZGSL peptide with affinities of approximately 3 pM, 73 pM and 12nM, which is in accordance with the binding of 6, 4 and 2 fingers respectively. 3x2F ZGLS show a similar trend in binding affinities. These experiments demonstrate that 3x2F peptides can bind contiguous 18 bp sites, but are also unique amongst the six finger peptides reported to date, in being able to bind sequences with two regions of unbound DNA with high affinity.

Binding Site Name	Binding Site Sequence*	Apparent Kd [†] (pM)			
		3x2F	3x2F	3x2F	3x2F
		ZGS	ZGL	ZGSL	ZGLS
123456 (bsC)	GCG GAC GCG GCG TGG GCG	0.6	0.9	ND	ND
12/34/56 (bsD)	GCG GAC T GCG GCG T TGG GCG	1.8×10^4	5	110	120
12//34//56 (bsE)	GCG GAC TC GCG GCG TC TGG GCG	ND	1.1×10^4	1.2×10^4	1.2×10^4
1234/56 (bsF)	GCG GAC T GCG GCG TGG GCG	54	ND	3	89
12/3456 (bsG)	GCG GAC GCG GCG T TGG GCG	77	ND	73	5

Table 5. The binding site sequences used in gel-shift experiments with the 3x2F peptides and the binding affinities determined. *Designed gaps in the target sequence are shown in bold. †ND (not done) represents experiments for which Kds are not calculated.

It appears that the more rigid nature of the 2x3F Zif-GAC peptide means that a mutation in the binding site of one finger is 'felt' only by that finger, so that the 123///56 site is bound with the extremely high affinity of 15 pM. In contrast, the results above show that the more sensitive design of the 3x2F peptides mean that a mutation

in the binding sequence of a single finger weakens the entire two-finger unit. Thus, the 3x2F ZGS peptide binds the same site with an affinity of 890 pM. The large reduction in affinity of the 3x2F ZGS peptide for the Zif268 half-site must be attributed to the extended linker sequence between fingers 2 and 3. Presumably this linker reduces the co-operative binding effect of the adjacent fingers, such that finger 3 of the peptide adds nothing to the binding of the half-site. Meanwhile, the unbound fingers probably 'drag' on the complex to help pull the peptide off the DNA. The higher affinity of the 3x2F peptides for other sites that are bound by only two fingers (such as the 3x2F ZGS peptide against the 12/34/56 site) presumably arises because there are three separate two-finger binding sites present in the sequence.

Example 14: Binding Affinities of Construct 3x1F ZIF

A peptide denoted 3x1F ZIF (Figure 8) is constructed by inserting a single glycine residue within each of the natural linkers in the wt ZIF gene. A further extension of this design is also used to create 6x1F ZG, which is a six-finger ZIF-GAC clone containing a glycine insertion within every linker peptide. The binding affinity of the 3x1F peptide for the 9bp ZIF site (bsA) is tested, and the construct is shown to bind the substrate.

Example 15. Structured Linkers

The experiments described in the following Examples seek to increase the utility of poly-zinc finger peptides by creating fusion peptides that are able to bind with high affinity to target sequences in which their binding subsites are separated by long (up to 10 bp) stretches of DNA. The Examples utilise structured linkers which are believed to show a preference for a particular length of DNA span, so that they maintain a high degree of specificity. The crystal structure of the first six fingers of TFIIIA bound to DNA (Nolte, R. T., Conlin, R. M., Harrison, S. C. & Brown, R. S. (1998) *Proc. Natl. Acad. Sci. USA* **95**, 2938-2943), indicate that that TFIIIA finger 4 may be a suitable candidate for a structured linker to span long (> 5 bp) stretches of DNA.

A fusion peptide comprising the first four fingers of TFIIIA and the three fingers of Zif268, called TF(1-4)-ZIF, is first created. This is shown to bind DNA with high affinity and showed a preference for sites containing 7 or 8 bps of non-bound DNA. In contrast, a similar construct that contains a 20 residue flexible linker, 5 TF(1-3)-flex-ZIF, is seen to bind its full-length target sites somewhat weaker. The data in these Examples suggests that TFIIIA finger 4 is a suitable 'structured' linker for spanning long stretches of DNA, and furthermore, that TF(1-4)-ZIF would make a good scaffold for 'designer' transcription factors that bind DNA with 7 or 8 bps of non-bound DNA.

10 The Examples also test the ability of a zinc finger module from Zif268 to act as a structured linker. A zinc finger from Zif268 is mutated to make it non sequence-specific, and then used to link the three wild-type fingers of Zif268 to a three-finger mutant of Zif268 (GAC). This 'serine-finger' is expected to sit in the major groove, spanning 3 or 4 bps of DNA. Surprisingly, this new peptide is found to be able to bind 15 with similar affinity to the continuous 18 bp sequence comprising the Zif268 and GAC sites, and to all the non-contiguous sites with 1-10 bp gaps. The fact that this peptide can bind tightly to the contiguous binding site and the sites with just 1 or 2 bp gaps suggests that the 'serine-finger' is able to flip out of the major groove to make space for the binding of its neighbouring fingers. This data indicates that within a zinc finger 20 array redundant fingers can make way for stronger DNA-binding domains. When the binding subsites are separated by 7-10 bps of DNA it is likely that the redundant finger lies across the surface of the DNA, in a manner analogous to TFIIIA finger 4 (15).

The Examples also describe a fusion construct, ZIF-F4-GAC, which uses 25 TFIIIA finger 4 as a linker between two Zif-type domains. This peptide displays little discrimination for the length of DNA span separating the binding subsites, although a trend in the binding affinities of the peptide is apparent. All peptides connected by zinc finger modules show a preference for sequences containing 3 bp or over 6 bp gaps. These probably correspond to binding modes when the zinc finger-linker is sat 'normally' in the major groove, or able to bridge the minor groove.

It has been proposed that the relatively hydrophobic linkers flanking TFIIIA finger 4 may constrain finger 4 into its orientation across the minor groove, as observed in the crystal structure of Nolte *et al.* (1998). Hence, the Examples also describe investigations into the conformational freedom of zinc fingers by swapping the linker sequences flanking wild-type TFIIIA finger 4 and the 'serine-finger'. It is found that the linker sequences flanking TFIIIA finger 4 only confer a small degree of structural rigidity, which is most apparent when the finger is forced to take up unfavourable conformations.

A predicted benefit of using structured linkers is that of increased binding affinity over peptides containing long, flexible linkers. This is confirmed by the Examples which disclose binding results from the two peptides containing 20 residue flexible linkers, which are found to bind their full-length targets between 3 and 10-fold weaker than peptides with structured linkers.

Poly-zinc finger peptides are likely to become increasingly important in gene therapy and the creation of transgenic organisms. Given the difficulty of engineering zinc finger peptides to bind to all possible DNA sequences (Choo, Y. & Klug, A. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 11168-11172; Segal, D. T., Dreier, B., Beerli, R. R. & Barbas, C. F. III (1999) *Proc. Natl. Acad. Sci. USA* **96**, 2758-2763.), it is advantageous to synthesise peptides capable of spanning long regions of DNA, while still binding with high affinity. This will allow the selection of favourable DNA target sites that may be several nucleotides apart. The Examples show that 'structured' linkers may be incorporated into zinc finger fusion peptides. These allow the separate DNA-binding domains to bind with high affinity to sites separated by 1 to 10 bps of non-bound DNA. The ability of these structured-linker fusion peptides to span such long stretches of DNA is particularly advantageous for the targeting of natural promoter sequences. For example, the zinc finger protein, Sp1, binds GC box DNA, which can appear in multiple copies in the promoter sequences upstream of a variety of cellular and viral genes (Kadonaga, J. T., Jones, K. A. & Tjian, R. (1986) *Trends Biochem. Sci.* **11**, 20-23; Bucher, P. (1990) *J. Mol. Biol.* **212**, 563-578). Similarly, the promoter for the HSV40 early genes contains three 21 bp repeats which include GC

boxes. Linking zinc finger peptides that recognise such regions could create powerful 'designer' transcription factors. TFIIIA finger 4 may be a particularly useful 'structured' linker as it shows a marked preference for 7 or 8 bp DNA spans.

The Examples also indicate that weakly binding zinc fingers are able to 'flip' in or out of the DNA major groove to accommodate neighbouring fingers within the DNA-binding domain. This means that certain zinc finger arrays will bind reasonably tightly to truncated or mutated binding sites. This feature of zinc-finger arrays may be taken advantage of, for instance to engineer zinc fingers which bind to a series of related, but different binding sites. Nature almost certainly takes advantage of this phenomenon to evolve zinc finger transcription factors that regulate multiple genes from non-identical promoters. Furthermore, many natural polydactyl proteins that have been isolated contain zinc fingers whose roles are not yet understood. For example, GL1 contains five tandem zinc fingers, but in the crystal structure of this protein only two of these bind to DNA in the classical -base specific- manner (Pavletich, N. P. & Pabo, C. O. (1991) *Science* **261**, 1701-1707). The results presented in the Examples also suggest that there may be a broad repertoire of roles for zinc finger domains within the cell. The Examples also show that polydactyl peptides comprising flexible linkers may be created that bind with far greater specificity than previously designed six-finger peptides.

Example 15A: Construction of TFIIIA(F1-4)-ZIF Zinc Finger Construct

The TFIIIA(F1-4) construct is made by fusing the first four fingers of TFIIIA N-terminally to the three fingers of wt ZIF. The natural linker between fingers 4 and 5 of TFIIIA is used as the linker between TFIIIA finger 4 and ZIF finger 1. However, the construct is designed such that the entire TFIIIA finger 4 region acts as a structured linker between TFIIIA fingers 1-3 (which bind DNA) and wt ZIF fingers 1-3 (which also bind DNA).

The construction of TFIIIA(F1-4) is described with reference to Figures 13 and 15. As shown in Figure 13, the TFIIIA(F1-4) construct is made by PCR using two

pairs of primers A + a and B + b to amplify wild type TFIIIA and wild type ZIF templates respectively. Primers A and b comprise restriction sites for *Nde*I and *Not*I respectively. The respective amplification products are then subjected to overlap PCR, with a template fill-in step. Finally, the products are amplified with end primers A + b,
 5 digested with *Not*I and *Nde*I, and ligated into *Not*I/*Nde*I digested pCITE-4b vector (Amersham International Plc).

Primer A (SEQ ID NO: 29):

10 5' ACT TCG GAA TTC GCG GCC CAG CCG GCC CAT ATG GGA GAG AAG GCG CTG CCG GTG 3' ^{*Nde* I}

Primer a (SEQ ID NO: 30):

15 5' GCA AGC ATA CGG CAG CTG CTG TGT GTG ACT G 3'

Primer B (SEQ ID NO: 31):

5' ACA CAG CAG CTG CCG TAT GCT TGC CCT GTC GAG TCC 3'

20 Primer b (SEQ ID NO: 32):

5' GAG TCA TTC AAG CTT TGC GGC CGC TTA GTC CTT CTG TCT TAA ATG GAT TTT GG 3' ^{*Not* I STOP}

Example 16: Construction of GAC-F4-ZIF Zinc Finger Construct

25 The GAC-F4-ZIF construct is made by joining the GAC-clone to the N-terminus of wt ZIF, using the entire TFIIIA finger 4 peptide (including its natural flanking linker sequences) as a structured linker.

The construction of GAC-F4-ZIF is described with reference to Figures 14 and 16. As shown in Figure 14, the GAC-F4-ZIF construct is made by PCR using two pairs
 30 of primers C + c and D + d to amplify the GAC clone and TFIIIA(F1-4) templates respectively. Primers C and d comprise restriction sites for *Nde*I and *Not*I respectively. The respective amplification products are then subjected to overlap PCR, with a template fill-in step. Finally, the products are amplified with end primers C + d,

digested with *NotI* and *NdeI*, and ligated into *NotI/NdeI* digested pCITE-4b vector (Amersham International Plc).

Primer C (SEQ ID NO: 33):

5' ACT TCG GAA TTC GCG GCC CAG CCG GCC CAT ^{Nde I} ATG GCA GAA CGC CCG TAT GCT TG 3'

Primer c (SEQ ID NO: 34):

10 5' CAC ATA GAC GCA GAT CTT GAT GTT ATG GAT TTT GGT ATG CCT CTT GCG 3'

Primer D (SEQ ID NO: 35):

5' CAT AAC ATC AAG ATC TGC GTC TAT GTG 3'

15 Primer d (SEQ ID NO: 36):

5' GAG TCA TTC AAG CTT TGC GGC CGC TTA ^{Not I} GTC CTT CTG TCT TAA ^{STOP} ATG GAT TTT GG 3'

Example 17: Construction of ZIF-ZnF-GAC Zinc Finger Construct

To create the ZIF-ZnF-GAC construct, primers A + b and C + d are used to
 20 amplify the wild type ZIF and GAC clone sequences, respectively. These are then
 digested with *EagI* to create sticky ends. Next, the "neutral" zinc finger (ZnF) is
 produced by annealing the following complimentary oligonucleotides: 5' GG CCG
 TTC CAG TGT CGA ATC TGC ATG CGT AAC TTC AGT TCT AGT AGC TCT
 CTT ACC AGC CAC ATC CGC ACC CAC ACA GGT GAG C 3' (SEQ ID NO: 37)
 25 and 5' GG CCG CTC ACC TGT GTG GGT GCG GAT GTG GCT GGT AAG AGA
 GCT ACT AGA ACT GAA GTT ACG CAT GCA GAT TCG ACA CTG GAA C 3'
 (SEQ ID NO:38), which create *EagI* sites at each end. The complete ZIF-ZnF-GAC
 construct is finally generated by joining the "neutral" finger to the *EagI* cut ZIF and
 GAC sequences. This construct is then digested with *NdeI* and *NotI* and ligated into
 30 similarly digested pCITE-4b vector (Amersham International Plc).

Example 17A. Construction of ZIF-F4-GAC, ZIF-F4mut-GAC, ZIF- mutZnF-GAC, TF(1-3)-flex-ZIF and ZIF-flex-GAC

ZIF-F4-GAC and ZIF-F4mut-GAC

The ZIF-F4-GAC and ZIF-F4mut-GAC constructs are made by three separate
5 PCR amplifications of the three fingers of Zif268, the three fingers of a Zif268 mutant peptide (GAC), and the fourth finger of TFIIIA. Two sequential overlap PCR reactions are then used to fuse the separate units together, creating seven-finger constructs.

ZIF-mutZnF-GAC

The ZIF-mutZnF-GAC construct is made by PCR amplification of the three-
10 fingers of wt Zif268 and the Zif268 mutant (GAC), creating *Eag* I sites at their C-and N-termini respectively. The structured linker, ZnF, described above in Example 17, is inserted between the *Eag* I cut ZIF and GAC three-finger units to create the complete seven-finger construct. The ZIF-mutZnF-GAC clone IS made by PCR amplification of the ZIF, GAC, and ZnF structured linker fragments to create mutant ends. These three
15 fragments are joined by two sequential rounds of overlap PCR as above.

TF(1-3)-flex-ZIF and ZIF-flex-GAC

The TF(1-3)-flex-ZIF and ZIF-flex-GAC constructs are created by PCR
amplification of the first three fingers of TFIIIA, the three fingers of Zif268 or the
three fingers of the GAC-clone - using appropriate oligonucleotides - which are
20 designed to generate the flexible 20 amino acid linker peptide, -TG(GSG)₅ERP-, and
Eag I sites at the position to be joined. The required six-finger constructs are
synthesised by digesting the PCR products with *Eag* I and ligating at that site. All
zinc-finger constructs are digested with *Xba* I and *Eco* RI restriction enzymes and
inserted into the similarly digested, eukaryotic expression vector pcDNA 3.1(-)
25 (Invitrogen). The sequences of all constructs are confirmed by dideoxy sequencing.

Example 18: Binding Affinities of Construct TFIIIA(F1-4)-ZIF

The initial study on a structured-linker containing fusion peptide is conducted on the TF(F1-4)-ZIF construct. This experiment is designed to investigate a couple of issues. First, can TFIIIA finger 4 be used, successfully, outside its natural protein
5 context, to bridge a region of DNA within a non-contiguous binding site? Second, to determine the optimal DNA span of TFIIIA finger 4 within a synthetic fusion peptide.

The TF(F1-4)-ZIF peptide is targeted against non-contiguous binding sites comprising the TFIIIA fingers 1-3 recognition site and the three-finger ZIF site, separated by between 5 and 10 bps of unbound DNA (Table 2). The relative affinity of
10 the peptide for these sites is then compared with its affinity for the ZIF subsite bsA alone. A selection of the gel shift results are shown in Figure 18, which shows that the TFIIIA(F1-4)-ZIF construct can bind nucleic acid substrates consisting of TFIIIA and ZIF subsites separated by 6 or 7 base pairs. From such gels it is clear that the DNA span of TFIIIA finger 4 in this construct is as much as 10bp. Non-contiguous binding
15 sites with 6-9 bps of intervening DNA can be bound, although the optimal spacing is found to be 7 or 8 bp. These optimal sites are bound at least 125-fold tighter than the ZIF site alone.

The results of this experiment accord with the fact that the fourth finger of TFIIIA is known not to bind DNA in a sequence-specific manner, and that this finger
20 jumps, spans or bridges the minor groove of DNA in the crystal structure of the first 6 fingers of TFIIIA (Nolte *et al.*, 1998, *Proc. Natl. Acad. Sci. USA* 95, 2938-2943.).

Example 19: Binding Affinities of Construct GAC-F4-ZIF

To determine whether TFIIIA F4 would still function as a linker when taken out of the context of neighbouring TFIIIA fingers, the GAC-F4-ZIF construct is made
25 (Figure 14 and 16). This construct can be thought of simply as two ZIF-based DNA binding domains joined by a structured linker (in this case TFIIIA F4). As above, this construct is tested for affinity against a range of sequences, comprising the appropriate

binding subsites separated by 6 to 11 bps of DNA (Table 3). In these studies TFIIIA finger 4 is again demonstrated to be an effective linker. Results of gel-shift experiments are shown in Figures 19 and 20. As before the new peptide is shown to bind its optimal, full-length target sites at least 125-fold stronger than the 9 bp ZIF site.

5 In this case, however, the optimal DNA span is found to be 8 or 9 bps, although 7-11 bp stretches could be spanned without a significant loss in binding affinity.

Example 20: Binding Affinities of Construct ZIF-ZnF-GAC

We next tested the possibility that a natural zinc finger, of the type found in the ZIF peptide, may function as a stable unit that spans 3 bps (or occasionally 4 bps) of

10 DNA while occupying the major groove. If so, a 'neutral' zinc finger module, i.e. one that does not recognise a specific DNA sequence, might be used as a structured linker to span 3 or 4 bps.

For this purpose a 'neutral' finger is created by replacing the DNA binding residues (those at positions -1, 2, 3, and 6) of wild type ZIF268 finger 2, with serine

15 residues. Serine can act as either an H-bond acceptor or donator, and can therefore interact with all four bases in DNA. This new finger, denoted "ZnF" and flanked by two GERP linkers, is used to join the three-finger peptides of ZIF and the GAC-clone, creating the seven-finger array ZIF-ZnF-GAC (Figure 17). This peptide is targeted against non-contiguous sites comprising the 9 bp ZIF and GAC-clone recognition

20 sequences separated by 2, 3, 4 or 5 bps of DNA, and also sites bsA and bsC for comparison (Figure 21). The results demonstrate that the peptide binds all full-length target sites comprising the ZIF and GAC subsites either adjacent or separated by up to 5 base pairs of unbound DNA at least 500-fold tighter than it does the ZIF site alone. These results suggest that the peptide may bind the contiguous ZIF-GAC site

25 fractionally weaker than it does the non-contiguous sites, but the difference (if any) is slight. Hence, it appears that the "neutral" zinc finger linker is able to function as an effective linker, either in or out of the DNA major groove.

Example 20A. Binding Affinity of TFIIIA / ZIF Fusion Peptides.

The TF(1-4)-ZIF and TF(1-3)-flex-ZIF peptides are tested against the non-contiguous TF-5,6,7,8,9-Z sites. In these first experiments the DNA composition of the non-bound region is based on the endogenous TFIIIA target site. The results clearly show that the TF(1-4)-ZIF peptide has a preference for non-contiguous sites separated by 7 or 8 bp gaps, which are bound with a K_d of approx. 3 pM (Table 6). The target sites with 5, 6 or 9 bp gaps are bound at least 5-fold weaker (Figure 27A). In contrast, the TF(1-3)-flex-ZIF peptide shows no preference for a particular DNA span, binding all non-contiguous sites with affinities of around 60 pM (Figure 27B). Further studies are conducted on binding sites with various sequences in the non-bound region of the DNA target site. These demonstrate that the peptides have no preference for particular sequence compositions within this non-bound region (data not shown). Both constructs bind the Zif268 half-site with similar affinity, as expected.

Example 20B. Binding Affinity of ZIF / GAC Fusion Peptides

The first binding study is conducted on ZIF-F4-GAC to determine the optimal span of TFIIIA finger 4 in this construct. This peptide is titrated against the continuous 18 bp ZM binding site, and non-continuous binding sites with 1-10 bps of non-bound DNA. Our results demonstrate that this peptide has little preference for a particular span of DNA, although the highest affinity binding is observed for sites containing 3 bp or ≥ 7 bp insertions (Table 7). The fact that this peptide is able to bind with such high affinity to sites with less than 3 bp gaps is highly unexpected. The slight reduction in binding affinity observed in these examples is presumably because the 1-2 bp gaps are too small to accommodate a zinc finger in the DNA major groove. In these circumstances it seems likely that the non-binding finger actually flips out of the DNA leaving the remaining fingers to bind the target site. The slight reduction in affinity for sites with 5 or 6 bp gaps is probably because TFIIIA finger 4 has to stretch half a helical turn around the DNA. For longer gaps the finger is likely to span the minor groove as is seen in wild-type TFIIIA.

A further set of binding studies is then carried out on the construct containing the non-specific zinc finger linker, ZIF-ZnF-GAC. Although, this construct is expected to target (primarily) non-contiguous sequences containing three or four base pairs of non-bound DNA, it is tested against all of the binding sites from ZM to Z10M. Our gel-shift data again demonstrates that this peptide is able to bind its optimal targets with very high affinity (3-4 pM), and shows a similar trend in binding affinity to the ZIF-F4-GAC peptide (Figure 27C). However, this peptide is able to bind its least favourable sites with slightly greater affinity than observed for the previous peptide (Table 7).

It was thought that the -NIKICV- and -TQQLP- linkers found either side of wild-type TFIIIA finger 4 would be more structured than the flexible -TGERP- linkers which flanked the serine-mutated finger of ZIF-ZnF-GAC. Therefore, the ZIF-mutF4-GAC and ZIF-mutZnF-GAC peptides are synthesised and tested to determine whether these linker sequences are responsible for the less selective binding of the ZIF-ZnF-GAC peptide. These new peptides are targeted against all eleven binding sequences, as above. The ZIF-mutZnF-GAC peptide is found to bind the Z5M and Z6M binding sites with K_d 's of 18 pM and 11 pM respectively. All other binding sites are bound with very similar affinities to the ZIF-ZnF-GAC peptide (data not shown). By comparison, the ZIF-mutF4-GAC peptide binds both the Z5M and Z6M sites with apparent K_d 's of 13 pM. From these data it appears that the -NIKICV- and -TQQLP- linkers slightly weaken the binding of the peptides to DNA sequences with 5 or 6 bp gaps. This may be because they are less flexible than the -TGERP- linkers, and are less able to bend around the DNA helix. No differences in DNA-binding characteristics for the different linker combinations are observed when the binding subsites are located on approximately the same face of the DNA.

Finally, the ZIF-flex-GAC peptide is examined in the same way as the structured-linker peptides above. This peptide, as with the TF(1-3)-flex-ZIF peptide, displays no preference for a particular length of DNA span, and bound all sites with affinities of approximately 50 pM. This 3-10 fold reduction in affinity - compared to peptides connected by structured linkers - is probably due to the increased

conformational freedom of this peptide, which makes DNA binding less entropically favourable.

Binding Site		Apparent Kd (pM)	
Name	Sequence*	TF(1-4)-ZIF	TF-flex-ZIF
ZIF	GCGTGGGCG	2000	1800
TF5Z	GCGTGGGCGX ₅ GGATGGGAGAC	21	63
TF6Z	GCGTGGGCGX ₆ GGATGGGAGAC	17	68
TF7Z	GCGTGGGCGX ₇ GGATGGGAGAC	3	57
TF8Z	GCGTGGGCGX ₈ GGATGGGAGAC	3	61
TF9Z	GCGTGGGCGX ₉ GGATGGGAGAC	15	58

Table 6. The binding site sequences used in gel-shift experiments with the TFI_{IIA}-ZIF fusion peptides and the binding affinities obtained. *Non-bound DNA bases in the target sequence are shown by a bold 'X'. The exact base composition of these gaps is found to have no significant effect on peptide affinity.

Binding Site		Apparent Kd (pM)	
Name	Sequence*	ZIF-F4-GAC	ZIF-ZnF-GAC
ZIF	GCGTGGGCG	2200	2000
ZM	GCGGACGCGGCGTGGGCG	11	7
Z1M	GCGGACGCGXGCGTGGGCG	6	4
Z2M	GCGGACGCGX ₂ GCGTGGGCG	7	6
Z3M	GCGGACGCGX ₃ GCGTGGGCG	5	4
Z4M	GCGGACGCGX ₄ GCGTGGGCG	13	3
Z5M	GCGGACGCGX ₅ GCGTGGGCG	16	8
Z6M	GCGGACGCGX ₆ GCGTGGGCG	17	7
Z7M	GCGGACGCGX ₇ GCGTGGGCG	5	3
Z8M	GCGGACGCGX ₈ GCGTGGGCG	5	6
Z9M	GCGGACGCGX ₉ GCGTGGGCG	5	4
Z10M	GCGGACGCGX ₁₀ GCGTGGGCG	4	3

Table 7. The binding site sequences used in gel-shift experiments with the ZIF-GAC fusion peptides and the binding affinities obtained. *Non-bound DNA bases in the target sequence are shown by a bold 'X'. The exact base composition of these gaps is found to have no significant effect on peptide affinity.

5 **Example 21: Binding Affinities of ZIF-GAC and 3x2F ZGS peptides to Targets with Deleted Subsequence**

This example shows the differential effects of looping out of a single finger from a zinc finger protein/DNA complex.

To investigate the effect of finger-flipping or looping in 2x3F and 3x2F zinc-
10 finger peptides, gel-shift experiments are conducted with the 2x3F ZIF-GAC peptide and the 3x2F ZGS peptide, against a selection of modified binding sites; bs1, bs2, bs3, bs4 (Figures 22 and 23), as well as bsA and bsC, as control sites. Figure 22 shows results of gel-shift experiments in which the 2x3F ZIF-GAC peptide is tested for binding to the 9 base pair ZIF binding site (target bsA), the 18 base pair ZIF-GAC
15 binding site (bsC) as well as bs1, bs2, bs3 and bs4, which comprise the ZIF-GAC bsC sequence, but with the three base subsequence recognised by finger 4 of 2x3F ZIF-GAC removed, and 0, 1, 2 or 3 base pairs respectively inserted in its place, while Figure 23 shows corresponding experiments using 3x2F ZGS peptide.

By comparing the relative affinities of each peptide for the sites bs1-4 against
20 the designed, full-length binding site, bsC; the ability of zinc-finger peptides to accommodate finger "flipping" can be demonstrated. The sequence of bs1 is similar to that of bsC, but with the three bases recognised by finger 4 of the 3x2F ZGS or 2x3F ZIF-GAC peptides completely removed. The sites bs2, bs3 and bs4 are identical to bs1, except for the insertion of 1, 2 or 3 base pairs (respectively), in the region
25 normally bound by zinc-finger 4 of the fusion peptides. The inserted residues are selected so that they would not be the same as the sequence recognised by finger 4. It should be noted that the binding site of bs4 is the same length as bsC, but zinc-finger 4

will not contribute binding energy to the complex with this site. The other sites, bs1, bs2 and bs3 are shorter by 3, 2 and 1 bps respectively.

The gel-shift results with the 2x3F ZIF-GAC and 3x2F ZGS peptides are shown in Figures 22 and 23 respectively. Serial 5-fold dilutions of peptide are made and incubated with 0.01 nM binding site. Significantly, the results demonstrate that the 3x2F ZGS peptide is far more selective for the correct, full-length binding site (bsC) than is the 2x3F ZIF-GAC peptide. The gel-shift results of Figure 23 show that the 3x2F ZGS peptide binds the incorrect, full-length binding site (bs4) approximately 125-fold weaker than it does bsC; its binding is therefore relatively specific. It also binds the site bs3 and bs2 with almost identical affinity to bs4. (These sites are truncated in the region normally bound by finger 4). The shortest site, bs1, is bound at least 625-fold less tightly than the correct binding sequence, bsC. The 3x2F ZGS peptide clearly binds bs1 slightly more tightly than it does the ZIF site alone, but the concentrations of protein and binding site used in these experiments are such that binding to the ZIF site alone is barely detectable. In contrast, the 2x3F ZIF-GAC peptide binds the sequence of bs4 only 5-fold more weakly than it does bsC, and as above, its affinity for the sites bs3 and bs2 are very similar to that of bs4, demonstrating that it is relatively non-specific. The peptide shows reasonable discrimination when targeted to the bs1 site, which it binds approximately 125-fold weaker than bsC. These data clearly demonstrate that the individual zinc-fingers within a zinc-finger array (such as the 2x3F ZIF-GAC and the 3x2F ZGS peptides) are able to "flip" out of the DNA major groove - when they do not recognise the DNA sequence presented to them - in order to allow the remaining zinc-fingers to bind in the most optimal conformation. The ability of the zinc-finger peptide to accommodate this conformational change is dependant on the construction of the peptide. These results show that the detrimental effects of finger "flipping" are far more pronounced in the 3x2F ZGS peptide than in the 2x3F ZIF-GAC peptide, demonstrating that 3x2F peptides are far more specific than 2x3F peptides.

Example 22. Use of Two Finger Library for Selection of Zinc Fingers

The simplest approach is to construct an isolated two finger library, comprising amino acids known to contribute to DNA-binding affinity and specificity. Such a library is constructed using suitable randomizations. A phage display library is
5 constructed using methods known in the art, and a number of 6-7 bp DNA targets are used in selections that are carried out essentially as detailed in patent applications WO 96/06166 and WO 98/53057. After the selection process is complete, a number of tightly binding zinc finger proteins are isolated.

Example 23. Use of Combinatorial Library for Selection of Zinc Fingers

10 We further demonstrate the construction of libraries for 2-finger domains whose register of interaction is precisely fixed. This is achieved by employing "GCG" anchors and two extensively-randomised zinc fingers. The libraries are designed to take into account synergistic effects between zinc fingers, by modifying cross-strand contacts from position 2. Consequently, position 2 of F2 is modified to Ser or Ala so
15 as to interact universally with either the 7'C in the "GCG" anchor, or any base (7'N) in the final target site sequence. Similarly, position 2 of F3 is modified to Ser or Ala so as not to interfere with the selection of bases 4'X or 4'X. Phage display libraries are constructed using methods known in the art, and a number of DNA targets are used in selections that are carried out essentially as detailed in patent applications WO
20 96/06166 and WO 98/53057. After the selection process is complete, a number of tightly binding zinc finger proteins are isolated. After selecting against particular DNA target sites, the genes for the appropriate 2-finger domains are easily recovered by PCR.

Example 24. Use of Combinatorial Library for Selection of Zinc Fingers

25 Phage Display libraries Lib1/2 and Lib 2/3 are used to select 2-Finger construction units. More specifically, the libraries are used to select two finger units that bind DNA sites of the form 5'-GXX XXX-3' or 5'-XXX XXG-3' (where X is any

base). Despite the fact that one base must be fixed as "G" in each target site, this still allows 2048 of all the 4096 ($=4^6$) possible 6-base 2-finger recognition sites to be targeted. Phage display libraries are constructed using methods known in the art, and a number of DNA targets are used in selections that are carried out essentially as
5 detailed in patent applications WO 96/06166 and WO 98/53057. After the selection process is complete, a number of tightly binding zinc finger proteins are isolated.

The genes for the appropriate 2-finger domains are easily recovered by PCR. Because of the design of the libraries, the "GCGG" or "GGCG" anchors serve to fix the register of DNA-protein interaction very precisely. Hence, the required 2-finger
10 domains may be specifically amplified from the respective libraries constructs by selective PCR using primers which bind only to the DNA sequence of finger 1 or finger 2 or finger 3. The first finger of the eventual 3x2F construct is preceded by an *Xba* I site and a MET codon. The second finger is joined to the third finger using an engineered *Eag* I site. The fourth finger is joined to the fifth finger through a *Bam*HI
15 site (at the end of finger 4) and a *Bgl* II site (at the start of finger 5). The sixth finger is followed by an *Eco*RI site.

The sequences are designed such that: If finger 2 joins to itself via the *Eag* I site, a *Not* I site is generated so this incorrect product can be recycled by digestion. When finger 4 joins correctly to finger 5 both *Bam*HI and *Bgl* II sites are destroyed,
20 however incorrectly fused units can be redigested with the appropriate enzyme. Hence, only the full-length 3x2F construct will be amplified with terminal primers following ligation of the three 2-finger units.

Using these construction techniques, the three 2-finger units selected as described above are fused to form a 3x2 protein.

25

Example 24. Library Selection of 2-Finger Units for Construction of 3x2f Peptides

As described above, 3x2F peptides may be made by linking 2 finger modules with suitable linkers. The above examples describe the isolation of such 2 finger

modules by ligation of synthetic oligonucleotides. However, and as described here, 2 finger modules may be selected by phage display using libraries (LIB12 and LIB23 libraries) comprising approximately one and a half fingers (see above and WO 98/53057).

5 Thus, the required 2-finger domains may be specifically amplified from the library constructs by selective PCR – using primers which bind only to the DNA sequence of finger 1 or finger 2 or finger 3. The sequences of these primers are as shown in the Examples above.

10 The first finger of the eventual 3x2F construct is preceded by an *Xba* I site and a MET codon. The second finger is joined to the third finger using an engineered *Eag* I site. The fourth finger is joined to the fifth finger through a *Bam*HI site (at the end of finger 4) and a *Bgl* II site (at the start of finger 5). The sixth finger is followed by an *Eco*RI site.

15 The sequences are designed such that: If finger 2 joins to itself via the *Eag* I site, a *Not* I site is generated so this incorrect product can be recycled by digestion. When finger 4 joins correctly to finger 5 both *Bam*HI and *Bgl* II sites are destroyed, however incorrectly fused units can be redigested with the appropriate enzyme. Hence, only the full-length 3x2F construct will be amplified with terminal primers following ligation of the three 2-finger units.

20

Example 25. Primer Sequences

25 Primers are named by the following method: A, B, C (in position 1) shows which of the three 2-finger units is to be amplified, A is the first two fingers of the 3x2F construct, B implies fingers 3 and 4 and C fingers 5 and 6. N, C (in position 2) shows whether the oligo primes from the N- or C-terminus. F1, F2, F3 shows which finger of the 3-finger library the primer binds to. L12, L23, L123 shows whether the primer binds specifically to LIB12, Lib23 or binds to both libraries.

The final two primers are specific for the extreme N- and C- termini of the 3x2F constructs and are used to amplify the full-length ligation product from any intermediate species.

- ANF1L12
 5 *Xba* I
 CAG TTG CGT **CTA GAC** GCC GCC **ATG** GCG GAG AGG CCC TAC GCA
 TGC
- ANF2L123
 10 *Xba* I
 CAG TTG CGT **CTA GAC** GCC GCC **ATG** GCT GAG AGG CCC TTC CAG
 TGT CGA ATC TGC AT
- ANF1L23
 15 *Xba* I
 CAG TTG CGT **CTA GAC** GCC GCC **ATG** GCA GAA CGC CCA TAT GCT
 TGC
- ACF3L12
 20 *Eag* I
 GC GGC CGC **CGG CCG** CTG GCC TCC TGT ATG GAT TTT GGT A
- ACF2L123
 25 *Eag* I
 CAT GGC ATT **CGG CCG** CTC GCC TCC TGT GTG GGT GCG GAT G
- ACF3L23
 30 *Eag* I
 GC GGC CGC **CGG CCG** TTG TCC GCC CGT GTG TAT CTT GGT A
- BNF1L12
 Eag I
 TCA AGC TGC **CGG CCG** TAC GCA TGC CCT GTC GAG TC
- BNF2L123
 35 *Eag* I
 AGC TCT CAG **CGG CCG** TTC CAG TGT CGA ATC TGC AT
- BNF1L23
 40 *Eag* I
 TCA AGC TGA **CGG CCG** TAT GCT TGC CCT GTC GAG TC
- BCF3L12
 45 *Bam*HI
 CGC GTC CTT CTG **GGA TCC** TGT ATG GAT TTT GGT A

BCF2L123
*Bam*H I
 ACC CTT CTC **GGA TCC** TGT GTG GGT GCG GAT G

5 BCF3L23
*Bam*H I
 C CGC ATC TTT TTG **GGA TCC** CGT GTG TAT CTT GGT A

CNF1L12
 10 *Bgl* II
 TCA AGC TGC **AGA TCT** GAG AGG CCC TAC GCA TGC CCT GTC

CNF2L123
 15 *Bgl* II
 ACG TCT ACG **AGA TCT** CAG AAG CCC TTC CAG TGT CGA ATC TGC AT

CNF1L23
Bgl II
 20 TCA AGC TGA **AGA TCT** GAA CGC CCA TAT GCT TGC CCT GTC

CCF3L12
*Eco*R I
 CAT **TTA GGA ATT** CCG GGC CGC GTC CTT CTG TCT CAG ATG GAT
 TTT

25 CCF2L123
*Eco*R I
 CAT **TTA GGA ATT** CCG GGC CGC ATC CTT CTG GCG CAG GTG GGT
 GCG GAT G

30 CCF3L23
*Eco*R I
 CAT **TTA GGA ATT** CCG GGC CGC ATC TTT TTG GCG CAG GTG TAT C

35 NXbaAMP
Xba I
 CAG TTG CGT **CTA GAC** GCC GCC

CEcoAMP
 40 *Eco*R I
 CAT TTA **GGA ATT** CCG GGC CGC

**Example 26. Selection of Sites and Construction of 3x2f Znf to Bind the GC Box /
 NRF-1 Site in Promoter Region of the CXCR4 Gene**

45 Promoter Sequence (top) with potential 6 bp sites marked below.

```

5'  TCCCCGCCCCAGCGGCGCATGCGCCGCGC 3'
A   TCCCCGCCCCAG  GGCGCA  GCGCCG
B           GCCCCAGCGGCGCATGCG
5   C           CAGCGGCGCATG

```

N.B. 6 bp sites are chosen which are either adjacent or within 1 bp of each other as 2-finger units bind optimally when within 1 bp of each other.

PROTOCOL

10 i) Select sites on row B.

Perform selections in usual manner. GCCCCA: target with LIB12 and take fingers 1 and 2 – F5+F6 of the 3x2 construct. GCGGCG: may be targeted by LIB12 and take fingers 1 and 2, or fingers 2 and 3; or may be targeted by LIB23 and take fingers 2 and 3 or fingers 1 and 2. Generates F3+F4 of the 3x2 construct. CATGCG:
 15 can be targeted by LIB23 and take fingers 2 and 3. Gives F1+F2 of the 3x2 construct.

ii) Join 2-finger units to create 3x2F peptide.

PCR amplify fingers binding appropriate sequences. Purify 2-finger products. Combine products, digest with *Eag* I, *Bam*H I and *Bgl* II. Heat inactivate *Eag* I. Ligate
 20 fragments together in the presence of *Not* I, *Bam*H I and *Bgl* II to destroy incorrectly ligated fragments. PCR amplify 6-finger construct with N – and C-terminal specific primers. Digest with *Xba* I and *Eco*R I, ligate into similarly digested vector – pTracer.

Example 27. Comparison of a 2x3F Peptide and a Similar 3x2F PeptideA. Creation of a 2x3F Peptide

3-finger units are selected to bind the 9-bp target sequences, **11** and **9** (below), essentially as described above and also in WO 98/53057.

5

```

11:      GCA GGG GTT
9:       GGC CAG GCG
11-9:    GGC CAG GCG GCA GGG GTT

```

The 3 finger peptide which binds site **11** is referred to as **pep11**, and the 3
 10 finger peptide which binds site **9** is referred to as **pep9**. To create a 2x3F peptide **pep11** is joined to the N-terminus of **pep9**, using the procedure below, and the new 6-finger construct is called **2x3F pep11-9**. This new peptide targets the contiguous sequence **11-9**, shown above.

All primer sequences in this Example are the same as the corresponding
 15 sequences in Example 25 having the same name. Primer CWT2 is identical to Primer a (SEQ ID NO: 2); Primer NWT3S is identical to Primer B (SEQ ID NO: 3); Primer CGAC1 is identical to Primer c (SEQ ID NO: 6); Primer NGAC2F is identical to Primer D (SEQ ID NO: 7). Primer 3x2CF3L23 has the following sequence: GC GGC CGC CGG CCG CTG GCC CGT GTG TAT CTT GGT A.

20 The sequence of 2x3Fpep11-9 is shown in Figure 27, and the sequence of 3x2Fpep11-9 is shown in Figure 28.

Construction Procedure

Primer pairs: **ANF1L12** and **BCF3L23**; and **CNF1L23** and **CCF3L23**, are used to amplify the DNA encoding **pep11** and **pep9** respectively. This created a *Bam*H
 25 I site at the 3' end of the **pep11** gene and a *Bgl* II site at the 5' end of the **pep9** gene. Hence, digestion of the PCR fragments with these enzymes, followed by ligation

created the 6-finger construct **2x3F pep11-9**, in which both original enzyme sites are destroyed and the peptide linker sequence –TGSERP- is created. The full-length fragment is then digested with *Xba* I and *EcoR* I and ligated into similarly digested pTracer (Invitrogen).

5 B. Creation of the 3x2F Peptide

To give a direct comparison between a selected 2x3F peptide and a 3x2F peptide targeted against the same DNA sequences, the zinc fingers of **pep11** and **pep9** are fused together in the style of a 3x2F peptide, using the procedure outlined below. This peptide, called **3x2F pep11-9**, targets the contiguous DNA sequence 11-9, above.

10 Again, primer and peptide sequences are as shown above and in the Figures.

Construction Procedure

Fingers 1 and 2 of **pep11** are amplified by PCR using primers **ANF1L12** and **CWT2**. Separately, finger 3 of **pep11** is amplified using primers **NWT3S** and **3x2CF3L23**. The 3-finger fragment **pep11(3x2)** is then created by overlap PCR using

15 the above fragments. Similarly, finger 1 of **pep9** is amplified using primers **BNF1L23** and **CGAC1**, and fingers 2 and 3 of **pep9** are amplified using primers **NGAC2S** and **CCF3L23**. The 3-finger fragment **pep9(3x2)** is then created by overlap PCR. The primers **3x2CF3L23** and **BNF1L23** produce *Eag* I restriction sites at the 3' and 5' ends of **pep11(3x2)** and **pep9(3x2)** respectively. Hence, digestion of the two 3-finger

20 fragments with *Eag* I, followed by ligation created the 6-finger construct **3x2F pep11-9**. In this peptide the linker sequences –TGGEKP- and –TGGQKP- are inserted between fingers 2 and 3 and fingers 4 and 5 respectively, and the sequence –TGQRP- separates fingers 3 and 4. The full-length fragment is then digested with *Xba* I and *EcoR* I and ligated into similarly digested pTracer (Invitrogen), as above.

C. Methods

The 2x3F pep11-9 and 3x2F pep11-9 peptides are compared by assessing their binding affinities for the 11-9 binding site and for binding site sequences mutated in the region bound by finger 1 (11-9mut1), finger 3 (11-9mut3), or with the bases
5 bound by finger 3 deleted (11-9del3). These sequences are shown below, with mutated regions underlined.

11-9: GGC CAG GCG GCA GGG GTT
11-9mut1: GGC CAG GCG GCA GGG ACC
10 11-9mut3: GGC CAG GCG ATG GGG GTT
11-9del3: GGC CAG GCG GGG GTT

In vitro fluorescence ELISA is used to estimate the binding specificity of each peptide for the various target sites, as described below.

Protocol for *In Vitro* Fluorescence ELISA

15 Preparation of Template

Zinc finger constructs are inserted into the protein expression vector pTracer (Invitrogen), downstream of the T7 RNA transcription promoter. Suitable templates for *in vitro* ELISA are created by PCR using the 5' primer (GCAGAGCTCTCTGGCTAACTAGAG), which binds upstream of the T7 promoter
20 and a 3' primer, which binds to the 3' end of the zinc finger construct and adds a sequence encoding for the HA-antibody epitope tag (YPYDVPDYA).

Zinc Finger Expression

In vitro transcription and translation are performed using the T7 TNT Quick Coupled Transcription / Translation System for PCR templates (Promega), according
25 to the manufacturers instructions, except that the medium is supplemented with 500 μ M ZnCl₂.

Fluorescence ELISA

DNA binding reactions contained the appropriate zinc finger peptide, biotinylated binding site (10 nM) and 5 µg competitor DNA (sonicated salmon sperm DNA), in a total volume of 50 µl, which contained: 1 x PBS (pH 7.0), 1.25×10^{-3} U high affinity anti-HA-Peroxidase antibody (Boehringer Mannheim), 50 µM ZnCl₂, 0.01 mg/ml BSA, and 0.5% Tween 20. Incubations are performed at room temperature for 40 minutes. Black streptavidin-coated wells are blocked with 4% marvel for 1 hour. Binding reactions are added to the streptavidin-coated wells and incubated for a further 40 minutes at room temperature. Wells are washed 5 times in 100 µl wash buffer (1 x PBS (pH 7.0), 50 µM ZnCl₂, 0.01 mg/ml BSA, and 0.5% Tween 20), and finally 50 µl QuantaBlu peroxidase substrate solution (Pierce) is added to detect bound HA-tagged zinc finger peptide. ELISA signals are read in a SPECTRAmax GeminiXS spectrophotometer (Molecular Devices) and analysed using SOFTmax Pro 3.1.2 (Molecular Devices).

D. Results

In Vitro Fluorescence ELISA Assay

To compare the specificity of the 2x3F pep11-9 and 3x2F pep11-9 peptides, samples from the same translation reaction are assayed against each of the binding sites above. The ELISA signals obtained from each assay are then normalised relative to the maximum signal obtained for that peptide. (In this way the absolute amount of either peptide produced by the *in vitro* transcription / translation system is insignificant). These data are then plotted on a graph, shown as Figure 26.

As can be seen, the data demonstrates that the 3x2F peptide shows greater selectivity / specificity for its correct target sequence, over mutant sequences, than does the 2x3F peptide.

Each of the applications and patents mentioned above, and each document cited or referenced in each of the foregoing applications and patents, including during the prosecution of each of the foregoing applications and patents ("application cited documents") and any manufacturer's instructions or catalogues for any products cited
5 or mentioned in each of the foregoing applications and patents and in any of the application cited documents, are hereby incorporated herein by reference. Furthermore, all documents cited in this text, and all documents cited or referenced in documents cited in this text, and any manufacturer's instructions or catalogues for any products cited or mentioned in this text, are hereby incorporated herein by reference.

10 Each of the applications and patents mentioned above, and each document cited or referenced in each of the foregoing applications and patents, including during the prosecution of each of the foregoing applications and patents ("application cited documents") and any manufacturer's instructions or catalogues for any products cited
15 or mentioned in each of the foregoing applications and patents and in any of the application cited documents, are hereby incorporated herein by reference. Furthermore, all documents cited in this text, and all documents cited or referenced in documents cited in this text, and any manufacturer's instructions or catalogues for any products cited or mentioned in this text, are hereby incorporated herein by reference. In particular, we hereby incorporate by reference International Patent Application
20 Numbers PCT/GB00/02080, PCT/GB00/02071, PCT/GB00/03765, United Kingdom Patent Application Numbers GB0001582.6, GB0001578.4, and GB9912635.1 as well as US09/478513.

Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the
25 scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended
30 to be within the scope of the following claims.

CLAIMS

1. A method of producing a modified nucleic acid binding polypeptide, the method comprising the steps of:

- 5 (a) providing a nucleic acid binding polypeptide comprising a plurality of nucleic acid binding modules;
- (b) selecting a first binding domain consisting of one or two contiguous nucleic acid binding modules;
- (c) selecting a second binding domain consisting of one or two contiguous nucleic acid binding modules; and
- 10 (d) introducing a linker sequence to link the first and second binding domains, the linker sequence comprising five or more amino acid residues.

2. A method of producing a modified nucleic acid binding polypeptide, the method comprising the steps of:

- 15 (a) providing a nucleic acid binding polypeptide comprising a plurality of nucleic acid binding modules;
- (b) selecting a first binding domain comprising a nucleic acid binding module;
- (c) selecting a second binding domain comprising a nucleic acid binding module; and
- 20 (d) introducing a linker sequence comprising a structured linker to link the first and second binding domains.

3. A method of producing a modified nucleic acid nucleic acid binding polypeptide, the method comprising the steps of:

- (a) providing a nucleic acid binding polypeptide comprising a plurality of nucleic acid binding modules;
- (b) selecting a first binding domain consisting of one or two contiguous nucleic acid binding modules;
- 5 (c) selecting a second binding domain consisting of one or two contiguous nucleic acid binding modules;
- (d) introducing a first linker sequence to link the first and second binding domains, the linker sequence comprising five or more amino acid residues;
- (e) selecting a third binding domain comprising a nucleic acid binding module;
- 10 (f) selecting a fourth binding domain comprising a nucleic acid binding module; and
- (g) introducing a second linker sequence comprising a structured linker to link the third and fourth binding domains.
4. A method according to any of Claim 1 or Claim 2, in which steps (b) to (d) are
15 repeated.
5. A method according to Claim 3, in which steps (b) to (d) and/or steps (e) to (g) are repeated.
6. A method according to any preceding claim, in which the binding affinity and/or specificity of the modified polypeptide to a nucleic acid sequence is increased
20 compared to the binding affinity and/or specificity of an unmodified polypeptide.
7. A method according to any preceding claim, in which the nucleic acid sequence comprises a sequence which is bound by the unmodified polypeptide.

8. A method according to any preceding claim, in which the nucleic acid sequence comprises a sequence bound by the unmodified nucleic acid binding polypeptide, into which one or more nucleic acid residues has been inserted.
9. A method according to any preceding claim, in which the nucleic acid residue(s) are inserted between target subsites bound by the first and second binding domains of the unmodified polypeptide.
10. A method according to Claim 8 or 9, in which the number of inserted nucleic acid residues is 5, 6, 7, 8, 9, 10 or 11.
11. A method of making a nucleic acid binding polypeptide, the method comprising the steps of:
- 10 (a) providing a first binding domain and a second binding domain, at least one of the first and second binding domains consisting of one or two nucleic acid binding module(s); and
- 15 (b) linking the first and second binding domains with a linker sequence comprising five or more amino acid residues.
12. A method of making a nucleic acid binding polypeptide, the method comprising the steps of:
- (a) providing a first binding domain comprising a nucleic acid binding module;
- (b) providing a second binding domain comprising a nucleic acid binding module; and
- 20 (c) linking the first and second binding domains with a linker sequence comprising a structured linker.

13. A method of making a nucleic acid binding polypeptide, the method comprising the steps of:
- (a) providing a first binding domain and a second binding domain, at least one of the first and second binding domains consisting of one or two nucleic acid binding module(s);
 - (b) linking the first and second binding domains with a first linker sequence comprising five or more amino acid residues;
 - (c) providing a third binding domain comprising a nucleic acid binding module;
 - (d) providing a fourth binding domain comprising a nucleic acid binding module; and
 - (e) linking the third and fourth binding domains with a second linker sequence comprising a structured linker.
14. A method according to Claim 1, 3 or 13, in which the first linker sequence comprises a flexible linker.
15. A nucleic acid binding polypeptide comprising a first binding domain and a second binding domain linked by a linker sequence comprising five or more amino acid residues, in which at least one of the first and second binding domains consists of one or two nucleic acid binding module(s).
16. A non-naturally occurring nucleic acid binding polypeptide comprising a first binding domain comprising a nucleic acid binding module and a second binding domain comprising a nucleic acid binding module, the first and second binding domains being linked by a linker sequence comprising a structured linker.

17. A nucleic acid binding polypeptide comprising a first binding domain comprising a nucleic acid binding module and a second binding domain comprising a nucleic acid binding module, the first and second binding domains being linked by a linker sequence comprising a structured linker; a third binding domain consisting of
5 one or two contiguous nucleic acid binding modules and a fourth binding domain consisting of one or two contiguous nucleic acid binding modules, the third and fourth binding domains being linked by a second linker sequence comprising five or more amino acid residues.
18. A method or polypeptide according to any preceding claim, in which the
10 nucleic acid binding module is a zinc finger of the Cys₂-His₂ type.
19. A method or polypeptide according to any preceding claim, in which the nucleic acid binding module is selected from the group consisting of naturally occurring zinc fingers and consensus zinc fingers.
20. A method or polypeptide according to any of Claims 1, 3, 11, 13, 15 or 17, in
15 which each of the first and the second binding domains consists of two binding modules.
21. A method or polypeptide according to any preceding claim, in which the first linker sequence comprises between 5 and 8 amino acid residues.
22. A method or polypeptide according to any preceding claim, in which the first
20 linker sequence is provided by insertion of one or more amino acid residues into a canonical linker sequence.
23. A method or polypeptide according to Claim 22, in which the canonical linker sequence is selected from GEKP, GERP, GQKP and GQRP.

24. A method or polypeptide according to any preceding claim, in which the first linker sequence comprises a sequence selected from: GGEKP, GGQKP, GGS¹GEKP, GGSGQKP, GGSGGSGEKP, and GGSGGSGQKP.
25. A method or polypeptide according to any preceding claim, in which the
5 nucleic acid binding polypeptide comprises a nucleic acid sequence selected from SEQ ID Nos: 22, 23, 24, 25, 26 and 27.
26. A method or polypeptide according to any preceding claim, in which the structured linker comprises an amino acid sequence which is not capable of specifically binding nucleic acid.
- 10 27. A method or polypeptide according to any preceding claim, in which the structured linker is derived from a zinc finger by mutation of one or more of its base contacting residues to reduce or abolish nucleic acid binding activity of the zinc finger.
28. A method or polypeptide according to any preceding claim, in which the structured linker comprises the amino acid sequence of TFIIIA finger IV.
- 15 29. A method or polypeptide according to any preceding claim, in which the zinc finger is finger 2 of wild type Zif268 mutated at positions -1, 2, 3 and 6.
30. A method or polypeptide according to any of Claims 2, 12, or 16, in which the first or second nucleic acid binding domain is selected from the group consisting of: fingers 1 to 3 of TFIIIA, GAC and Zif, or a method or polypeptide according to any of
20 Claims 3, 13 or 17, in which the third or fourth nucleic acid binding domain is selected from said group.
31. A method or polypeptide according to any preceding claim, in which the nucleic acid binding polypeptide comprises substantially the sequence of Zif-ZnF-

GAC (SEQ ID NO: 55), GAC-F4-Zif (SEQ ID NO: 54) or TF(1-4)-ZIF (SEQ ID NO: 53).

32. A method or polypeptide according to any preceding claim, in which the or each linker sequence comprises one or more further sequence(s), each further sequence
5 comprising a canonical linker sequence, preferably GEKP, GERP, GQKP or GQRP, optionally comprising one or more amino acid sequences inserted into the canonical sequence.

33. A method or polypeptide according to Claim 32, in which said further sequences are selected from: GGEKP, GGQKP, GGSGEKP, GGSGQKP,
10 GGSGGSGEKP, and GGSGGSGQKP.

34. A nucleic acid binding polypeptide produced by a method according to any of Claims 1 to 14 and 18 to 33.

35. A nucleic acid encoding a nucleic acid binding polypeptide according to any of Claims 15 to 33.

15 36. A host cell transformed with a nucleic acid according to Claim 35.

37. A pharmaceutical composition comprising a polypeptide according to any of Claims 15 to 33 or a nucleic acid according to Claim 35 together with a pharmaceutically acceptable carrier.

38. Use of a structured linker in a method of making a nucleic acid binding
20 polypeptide.

39. Use according to Claim 38, in which the structured linker separates first and second nucleic acid binding domains of the nucleic acid binding polypeptide, to enable

the polypeptide to bind a nucleic acid target in which subsites bound by respective domains of the polypeptide are separated by one or more nucleic acid residues.

40. A nucleic acid binding polypeptide comprising a repressor domain and a plurality of nucleic acid binding domains, the nucleic acid binding domains being
5 linked by at least one non-canonical linker.
41. A nucleic acid binding polypeptide according to Claim 40, in which the repressor domain is a transcriptional repressor domain selected from the group consisting of: a KRAB-A domain, an *engrailed* domain and a *snag* domain.
42. A nucleic acid binding polypeptide according to Claim 40 or 41, in which the
10 nucleic acid binding domains are linked by at least one flexible linker.
43. A nucleic acid binding polypeptide according to Claim 40, 41 or 42, in which the nucleic acid binding domains are linked by at least one structured linker.
44. Use of a nucleic acid binding domain comprising two zinc finger modules as a basic unit in the construction of a nucleic acid binding polypeptide.
- 15 45. A method of producing a nucleic acid binding polypeptide, the method comprising providing a first and a second nucleic acid binding domain each comprising two zinc finger modules, and linking the first and second nucleic acid binding domains with a structured linker sequence or a flexible linker sequence.
- 20 46. Use of a amino acid sequence comprising five or more amino acid residues as a flexible linker to join two or more nucleic acid binding domains comprising two zinc finger modules.

47. Use of an amino acid sequence comprising a zinc finger which is not capable of specifically binding nucleic acid, as a structured linker to join two or more nucleic acid binding domains comprising two zinc finger modules.
48. Use according to Claim 44, 46 or 47 or a method according to Claim 45, in which the nucleic acid binding domain is selected from a zinc finger polypeptide library, in which each polypeptide in the library comprises more than one zinc finger and wherein each polypeptide has been at least partially randomised such that the randomisation extends to cover the overlap of a single pair of zinc fingers.
49. A method for producing nucleic acid binding domains comprising two zinc finger modules for use in constructing a nucleic acid binding polypeptide, the method comprising the steps of:
- (a) providing a zinc finger polypeptide library, in which each polypeptide in the library comprises more than one zinc finger and wherein each polypeptide has been at least partially randomised such that the randomisation extends to cover the overlap of a single pair of zinc fingers;
 - (b) providing a nucleic acid sequence comprising at least 6 nucleotides; and
 - (c) selecting sequences in the zinc finger library which are capable of binding to the nucleic acid sequence.
50. A use or method according to Claim 48, or a method according to Claim 49, in which substantially one and a half zinc fingers are randomised in each polypeptide.
51. A nucleic acid binding polypeptide comprising units of zinc finger binding domains linked by flexible and/or structured linkers, each zinc finger binding domain comprising two zinc finger modules.

1 / 27

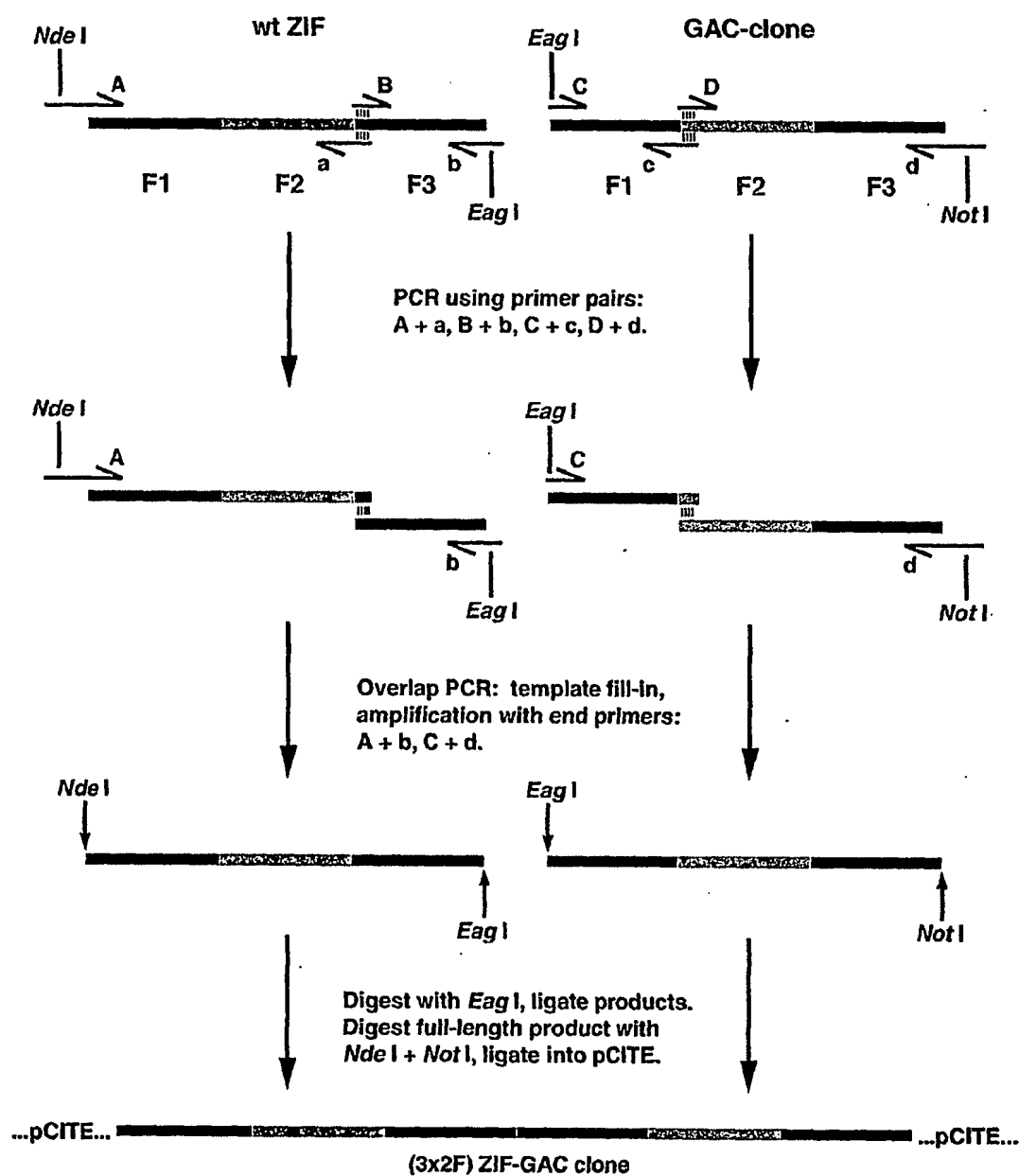


FIG. 1

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ZIF-GAC

SEQ ID NO: 21

1/1 31/11
 ATG GCA GAA CGC CCG TAT GCT TGC CCT GTC GAG TCC TGC GAT CGC CGC TTT TCT CGC TCG
 M A E R P Y A C P V E S C D R R F S R S
 61/21 91/31
 GAT GAG CTT ACC CGC CAT ATC CGC ATC CAC ACA GGC CAG AAG CCC TTC CAG TGT CGA ATC
 D E L T R H I R I H T G Q K P F Q C R I
 121/41 151/51
 TGC ATG CGT AAC TTC AGT CGT AGT GAC CAC CTT ACC ACC CAC ATC CGC ACC CAC ACA GGC
 C M R N F S R S D H L T T H I R T H T G
 181/61 211/71
 GAG AAG CCT TTT GCC TGT GAC ATT TGT GGG AGG AAG TTT GCC AGG AGT GAT GAA CGC AAG
 E K P F A C D I C G R K F A R S D E R K
 241/81 271/91
 AGG CAT ACC AAA ATC CAT TTA AGA CAG AAG GAC GGC GAA CGG CCG TAT GCT TGC CCT GTC
 R H T K I H L R Q K D G E R P Y A C P V
 301/101 331/111
 GAG TCC TGC GAT CGC CGC TTT TCT CGC TCG GAT GAG CTT ACC CGC CAT ATC CGC ATC CAC
 E S C D R R F S R S D E L T R H I R I H
 361/121 391/131
 ACA GGC CAG AAG CCC TTC CAG TGT CGA ATC TGC ATG CGT AAC TTC AGT GAT AGA AGC AAT
 T G Q K P F Q C R I C M R N F S D R S N
 421/141 451/151
 CTT GAA CGT CAC ACG AGG ACC CAC ACA GGC GAG AAG CCT TTT GCC TGT GAC ATT TGT GGG
 L E R H T R T H T G E K P F A C D I C G
 481/161 511/171
 AGG AAG TTT GCC AGG AGT GAT GAA CGC AAG AGG CAT ACC AAA ATC CAT TTA AGA CAG AAG
 R K F A R S D E R K R H T K I H L R Q K
 541/181
 GAC
 D

FIG. 2

3 / 27

3x2F ZGS

SEQ ID NO: 22

```

1/1                               31/11
ATG GCA GAA CGC CCG TAT GCT TGC CCT GTC GAG TCC TGC GAT CGC CGC TTT TCT CGC TCG
M  A  E  R  P  Y  A  C  P  V  E  S  C  D  R  R  F  S  R  S
61/21                             91/31
GAT GAG CTT ACC CGC CAT ATC CGC ATC CAC ACA GGC CAG AAG CCC TTC CAG TGT CGA ATC
D  E  L  T  R  H  I  R  I  H  T  G  Q  K  P  F  Q  C  R  I
121/41                           151/51
TGC ATG CGT AAC TTC AGT CGT AGT GAC CAC CTT ACC ACC CAC ATC CGC ACC CAC ACA GGT
C  M  R  N  F  S  R  S  D  H  L  T  T  H  I  R  T  H  T  G
181/61                           211/71
GGC GAG AAG CCT TTT GCC TGT GAC ATT TGT GGG AGG AAG TTT GCC AGG AGT GAT GAA CGC
G  E  K  P  F  A  C  D  I  C  G  R  K  F  A  R  S  D  E  R
241/81                           271/91
AAG AGG CAT ACC AAA ATC CAT ACC GGT GAA CGG CCG TAT GCT TGC CCT GTC GAG TCC TGC
K  R  H  T  K  I  H  T  G  E  R  P  Y  A  C  P  V  E  S  C
301/101                          331/111
GAT CGC CAC TTT TCT CGC TCG GAT GAG CTT ACC CGC CAT ATC CGC ATC CAC ACA GGT GGC
D  R  H  F  S  R  S  D  E  L  T  R  H  I  R  I  H  T  G  G
361/121                          391/131
CAG AAG CCC TTC CAG TGT CGA ATC TGC ATG CGT AAC TTC AGT GAT AGA AGC AAT CTT GAA
Q  K  P  F  Q  C  R  I  C  M  R  N  F  S  D  R  S  N  L  E
421/141                          451/151
CGT CAC ACG AGG ACC CAC ACA GGC GAG AAG CCT TTT GCC TGT GAC ATT TGT GGG AGG AAG
R  H  T  R  T  H  T  G  E  K  P  F  A  C  D  I  C  G  R  K
481/161                          511/171
TTT GCC AGG AGT GAT GAA CGC AAG AGG CAT ACC AAA ATC CAT TTA AGA CAG AAG GAC
F  A  R  S  D  E  R  K  R  H  T  K  I  H  L  R  Q  K  D

```

FIG. 3

4 / 27

3x2F ZGL

SEQ ID NO: 23

```

1/1                               31/11
ATG GCA GAA CGC CCG TAT GCT TGC CCT GTC GAG TCC TGC GAT CGC CGC TTT TCT CGC TCG
M  A  E  R  P  Y  A  C  P  V  E  S  C  D  R  R  F  S  R  S
61/21                             91/31
GAT GAG CTT ACC CGC CAT ATC CGC ATC CAC ACA GGC CAG AAG CCC TTC CAG TGT CGA ATC
D  E  L  T  R  H  I  R  I  H  T  G  Q  K  P  F  Q  C  R  I
121/41                          151/51
TGC ATG CGT AAC TTC AGT CGT AGT GAC CAC CTT ACC ACC CAC ATC CGC ACC CAC ACA GGC
C  M  R  N  F  S  R  S  D  H  L  T  T  H  I  R  T  H  T  G
181/61                          211/71
GGT TCT GGC GAG AAG CCT TTT GCC TGT GAC ATT TGT GGG AGG AAG TTT GCC AGG AGT GAT
G  S  G  E  K  P  F  A  C  D  I  C  G  R  K  F  A  R  S  D
241/81                          271/91
GAA CGC AAG AGG CAT ACC AAA ATC CAT ACC GGT GAA CGG CCG TAT GCT TGC CCT GTC GAG
E  R  K  R  H  T  K  I  H  T  G  E  R  P  Y  A  C  P  V  E
301/101                        331/111
TCC TGC GAT CGC CAC TTT TCT CGC TCG GAT GAG CTT ACC CGC CAT ATC CGC ATC CAC ACA
S  C  D  R  H  F  S  R  S  D  E  L  T  R  H  I  R  I  H  T
361/121                        391/131
GGC GGT TCT GGC CAG AAG CCC TTC CAG TGT CGA ATC TGC ATG CGT AAC TTC AGT GAT AGA
G  G  S  G  Q  K  P  F  Q  C  R  I  C  M  R  N  F  S  D  R
421/141                        451/151
AGC AAT CTT GAA CGT CAC ACG AGG ACC CAC ACA GGC GAG AAG CCT TTT GCC TGT GAC ATT
S  N  L  E  R  H  T  R  T  H  T  G  E  K  P  F  A  C  D  I
481/161                        511/171
TGT GGG AGG AAG TTT GCC AGG AGT GAT GAA CGC AAG AGG CAT ACC AAA ATC CAT TTA AGA
C  G  R  K  F  A  R  S  D  E  R  K  R  H  T  K  I  H  L  R
541/181
CAG AAG GAC
Q  K  D

```

FIG. 4

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3x2F ZGXL

SEQ ID NO: 24

1/1 31/11
 ATG GCA GAA CGC CCG TAT GCT TGC CCT GTC GAG TCC TGC GAT CGC CGC TTT TCT CGC TCG
 M A E R P Y A C P V E S C D R R F S R S
 61/21 91/31
 GAT GAG CTT ACC CGC CAT ATC CGC ATC CAC ACA GGC CAG AAG CCC TTC CAG TGT CGA ATC
 D E L T R H I R I H T G Q K P F Q C R I
 121/41 151/51
 TGC ATG CGT AAC TTC AGT CGT AGT GAC CAC CTT ACC ACC CAC ATC CGC ACC CAC ACA GGC
 C M R N F S R S D H L T T H I R T H T G
 181/61 211/71
GGT TCT GGC GGT TCT GGC GAG AAG CCT TTT GCC TGT GAC ATT TGT GGG AGG AAG TTT GCC
 G S G G S G E K P F A C D I C G R K F A
 241/81 271/91
 AGG AGT GAT GAA CGC AAG AGG CAT ACC AAA ATC CAT ACC GGT GAA CGG CCG TAT GCT TGC
 R S D E R K R H T K I H T G E R P Y A C
 301/101 331/111
 CCT GTC GAG TCC TGC GAT CGC CAC TTT TCT CGC TCG GAT GAG CTT ACC CGC CAT ATC CGC
 P V E S C D R H F S R S D E L T R H I R
 361/121 391/131
 ATC CAC ACA GGC GGT TCT GGC GGT TCT GGC CAG AAG CCC TTC CAG TGT CGA ATC TGC ATG
 I H T G G S G G S G Q K P F Q C R I C M
 421/141 451/151
 CGT AAC TTC AGT GAT AGA AGC AAT CTT GAA CGT CAC ACG AGG ACC CAC ACA GGC GAG AAG
 R N F S D R S N L E R H T R T H T G E K
 481/161 511/171
 CCT TTT GCC TGT GAC ATT TGT GGG AGG AAG TTT GCC AGG AGT GAT GAA CGC AAG AGG CAT
 P F A C D I C G R K F A R S D E R K R H
 541/181 571/191
 ACC AAA ATC CAT TTA AGA CAG AAG GAC
 T K I H L R Q K D

FIG. 5

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3x2F ZGSL

SEQ ID NO: 25

1/1 31/11
 ATG GCA GAA CGC CCG TAT GCT TGC CCT GTC GAG TCC TGC GAT CGC CGC TTT TCT CGC TCG
 M A E R P Y A C P V E S C D R R F S R S
 61/21 91/31
 GAT GAG CTT ACC CGC CAT ATC CGC ATC CAC ACA GGC CAG AAG CCC TTC CAG TGT CGA ATC
 D E L T R H I R I H T G Q K P F Q C R I
 121/41 151/51
 TGC ATG CGT AAC TTC AGT CGT AGT GAC CAC CTT ACC ACC CAC ATC CGC ACC CAC ACA GGT
 C M R N F S R S D H L T T H I R T H T G
 181/61 211/71
GGC GAG AAG CCT TTT GCC TGT GAC ATT TGT GGG AGG AAG TTT GCC AGG AGT GAT GAA CGC
 G E K P F A C D I C G R K F A R S D E R
 241/81 271/91
 AAG AGG CAT ACC AAA ATC CAT ACC GGT GAA CGG CCG TAT GCT TGC CCT GTC GAG TCC TGC
 K R H T K I H T G E R P Y A C P V E S C
 301/101 331/111
 GAT CGC CAC TTT TCT CGC TCG GAT GAG CTT ACC CGC CAT ATC CGC ATC CAC ACA GGC GGT
 D R H F S R S D E L T R H I R I H T G G
 361/121 391/131
TCT GGC CAG AAG CCC TTC CAG TGT CGA ATC TGC ATG CGT AAC TTC AGT GAT AGA AGC AAT
 S G Q K P F Q C R I C M R N F S D R S N
 421/141 451/151
 CTT GAA CGT CAC ACG AGG ACC CAC ACA GGC GAG AAG CCT TTT GCC TGT GAC ATT TGT GGG
 L E R H T R T H T G E K P F A C D I C G
 481/161 511/171
 AGG AAG TTT GCC AGG AGT GAT GAA CGC AAG AGG CAT ACC AAA ATC CAT TTA AGA CAG AAG
 R K F A R S D E R K R H T K I H L R Q K
 541/181
 GAC
 D

FIG. 6

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3x2F ZGLS

SEQ ID NO: 26

1/1 31/11
ATG GCA GAA CGC CCG TAT GCT TGC CCT GTC GAG TCC TGC GAT CGC CGC TTT TCT CGC TCG
M A E R P Y A C P V E S C D R R F S R S
61/21 91/31
GAT GAG CTT ACC CGC CAT ATC CGC ATC CAC ACA GGC CAG AAG CCC TTC CAG TGT CGA ATC
D E L T R H I R I H T G Q K P F Q C R I
121/41 151/51
TGC ATG CGT AAC TTC AGT CGT AGT GAC CAC CTT ACC ACC CAC ATC CGC ACC CAC ACA GGC
C M R N F S R S D H L T T H I R T H T G
181/61 211/71
GGT TCT GGC GAG AAG CCT TTT GCC TGT GAC ATT TGT GGG AGG AAG TTT GCC AGG AGT GAT
G S G E K P F A C D I C G R K F A R S D
241/81 271/91
GAA CGC AAG AGG CAT ACC AAA ATC CAT ACC GGT GAA CGG CCG TAT GCT TGC CCT GTC GAG
E R K R H T K I H T G E R P Y A C P V E
301/101 331/111
TCC TGC GAT CGC CAC TTT TCT CGC TCG GAT GAG CTT ACC CGC CAT ATC CGC ATC CAC ACA
S C D R H F S R S D E L T R H I R I H T
361/121 391/131
GGT GGC CAG AAG CCC TTC CAG TGT CGA ATC TGC ATG CGT AAC TTC AGT GAT AGA AGC AAT
G G Q K P F Q C R I C M R N F S D R S N
421/141 451/151
CTT GAA CGT CAC ACG AGG ACC CAC ACA GGC GAG AAG CCT TTT GCC TGT GAC ATT TGT GGG
L E R H T R T H T G E K P F A C D I C G
481/161 511/171
AGG AAG TTT GCC AGG AGT GAT GAA CGC AAG AGG CAT ACC AAA ATC CAT TTA AGA CAG AAG
R K F A R S D E R K R H T K I H L R Q K
541/181
GAC
D

FIG. 7

8 / 27

3x1F ZIF

SEQ ID NO: 27

```
1/1                               31/11
ATG GCA GAA CGC CCG TAT GCT TGC CCT GTC GAG TCC TGC GAT CGC CGC TTT TCT CGC TCG
M  A  E  R  P  Y  A  C  P  V  E  S  C  D  R  R  F  S  R  S
61/21                             91/31
GAT GAG CTT ACC CGC CAT ATC CGC ATC CAC ACA GGT GGC CAG AAG CCC TTC CAG TGT CGA
D  E  L  T  R  H  I  R  I  H  T  G  G  Q  K  P  F  Q  C  R
121/41                          151/51
ATC TGC ATG CGT AAC TTC AGT CGT AGT GAC CAC CTT ACC ACC CAC ATC CGC ACC CAC ACA
I  C  M  R  N  F  S  R  S  D  H  L  T  T  H  I  R  T  H  T
181/61                          211/71
GGT GGC GAG AAG CCT TTT GCC TGT GAC ATT TGT GGG AGG AAG TTT GCC AGG AGT GAT GAA
G  G  E  K  P  F  A  C  D  I  C  G  R  K  F  A  R  S  D  E
241/81                          271/91
CGC AAG AGG CAT ACC AAA ATC CAT TTA AGA CAG AAG GAC
R  K  R  H  T  K  I  H  L  R  Q  K  D
```

FIG. 8

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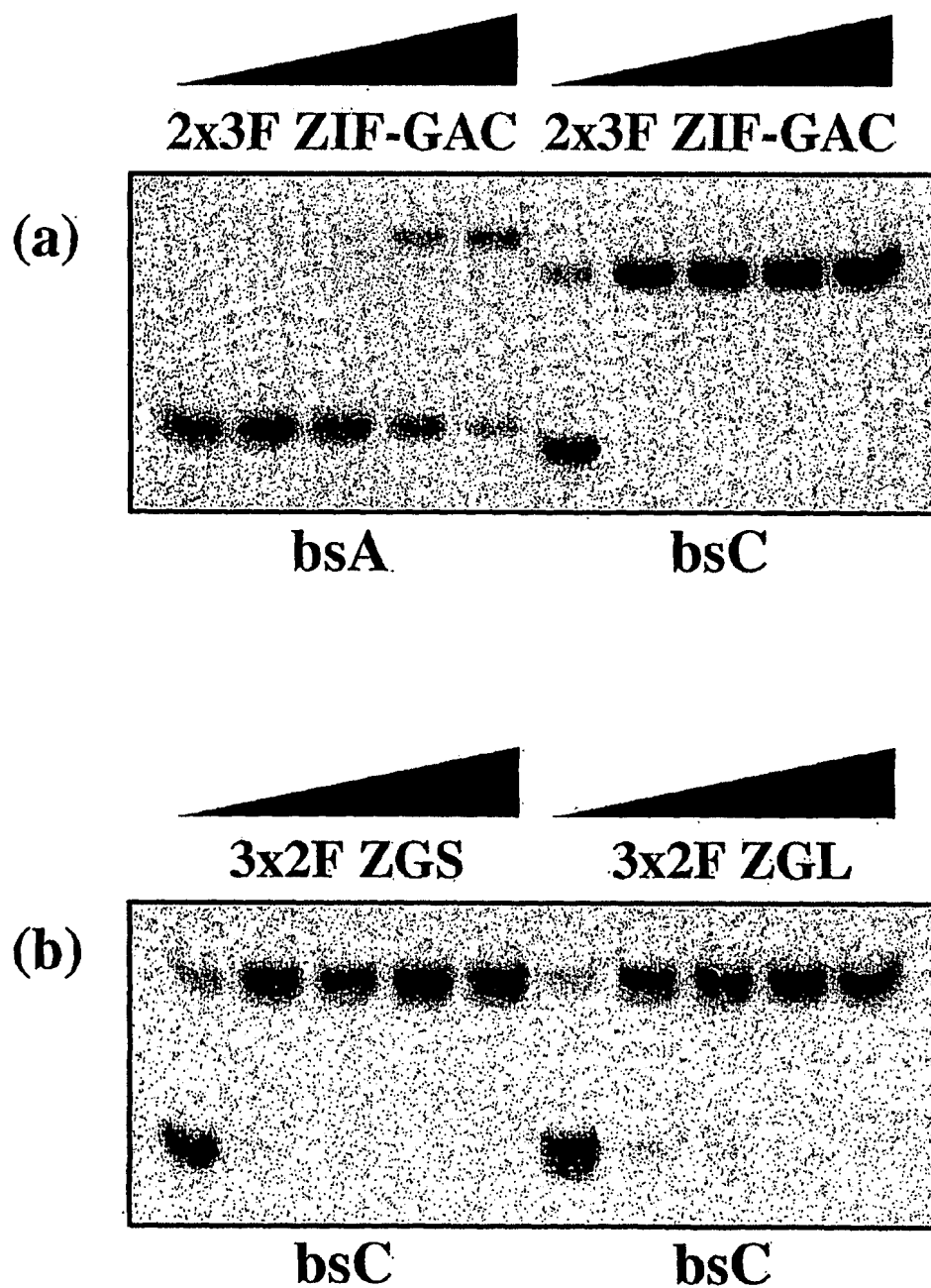


FIG. 9

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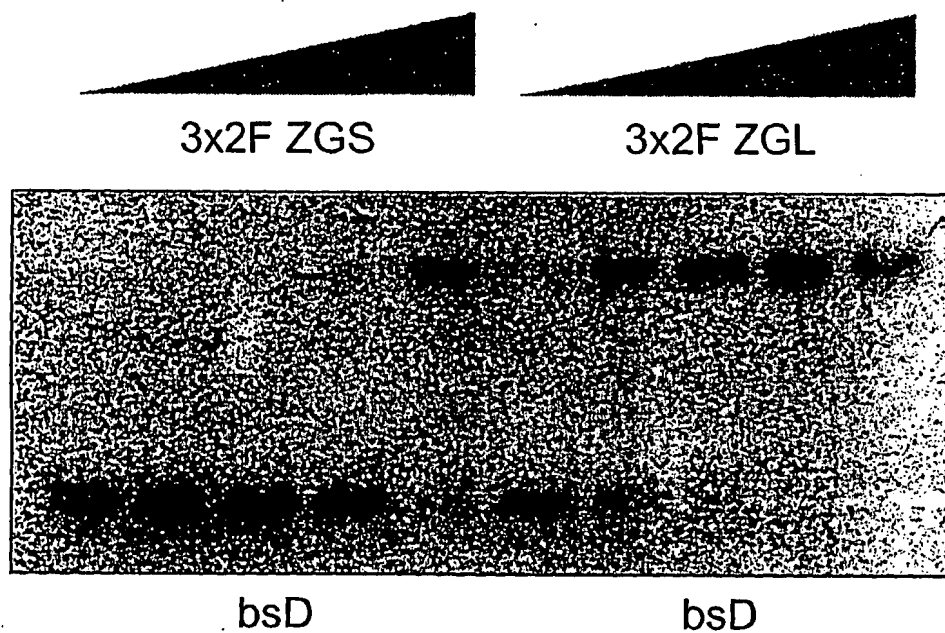


FIG. 10

11 / 27

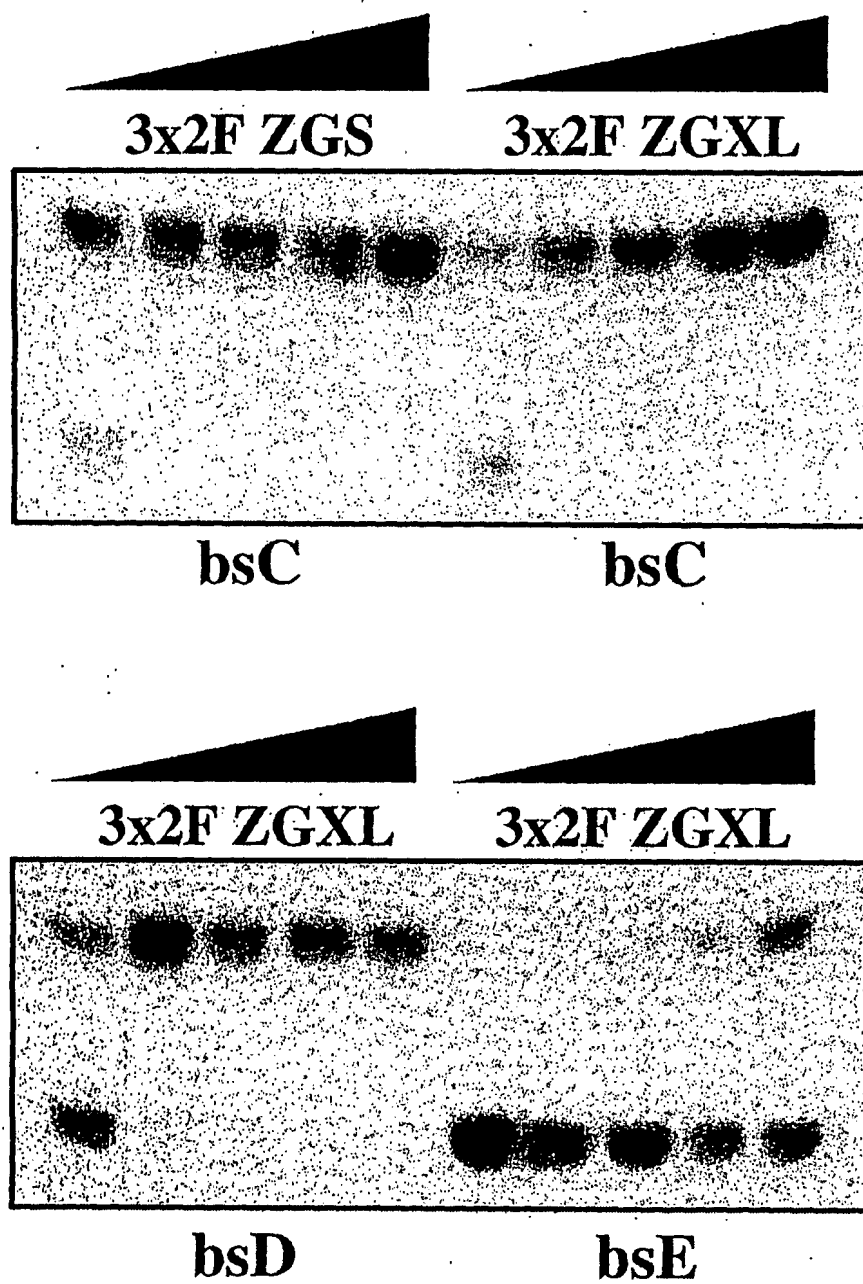


FIG. 11

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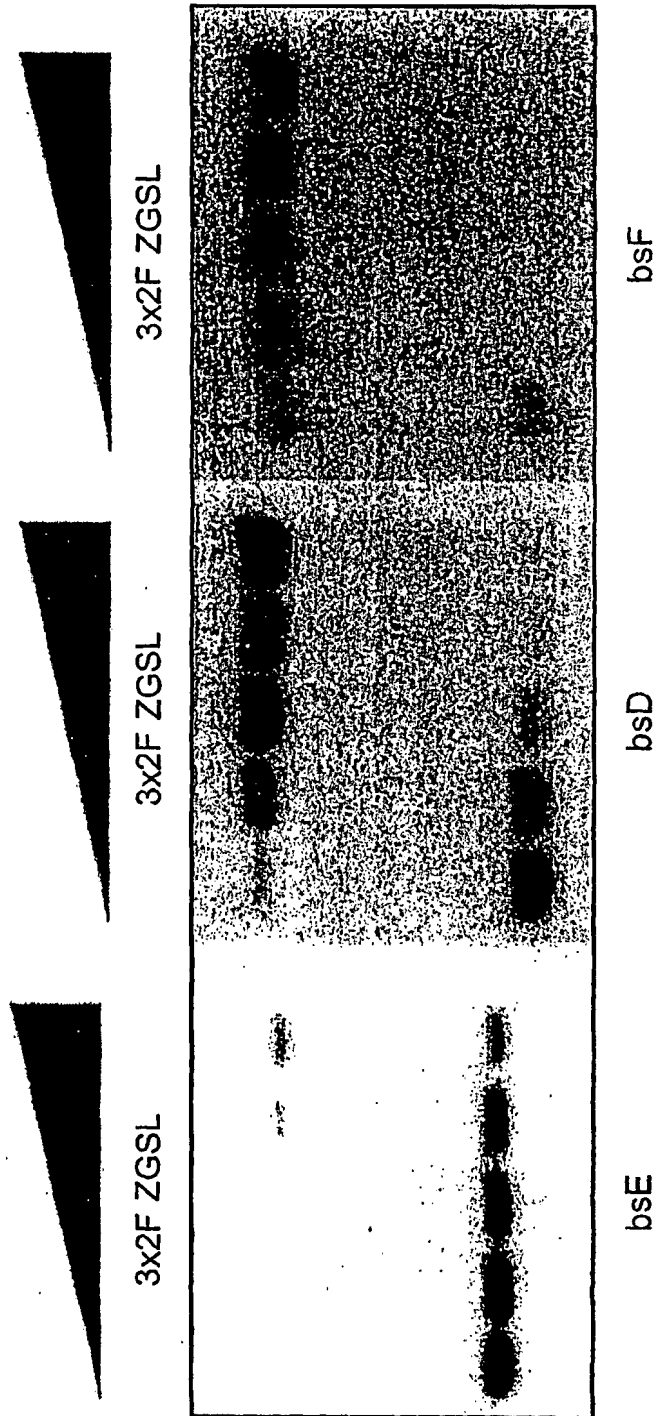


FIG. 12

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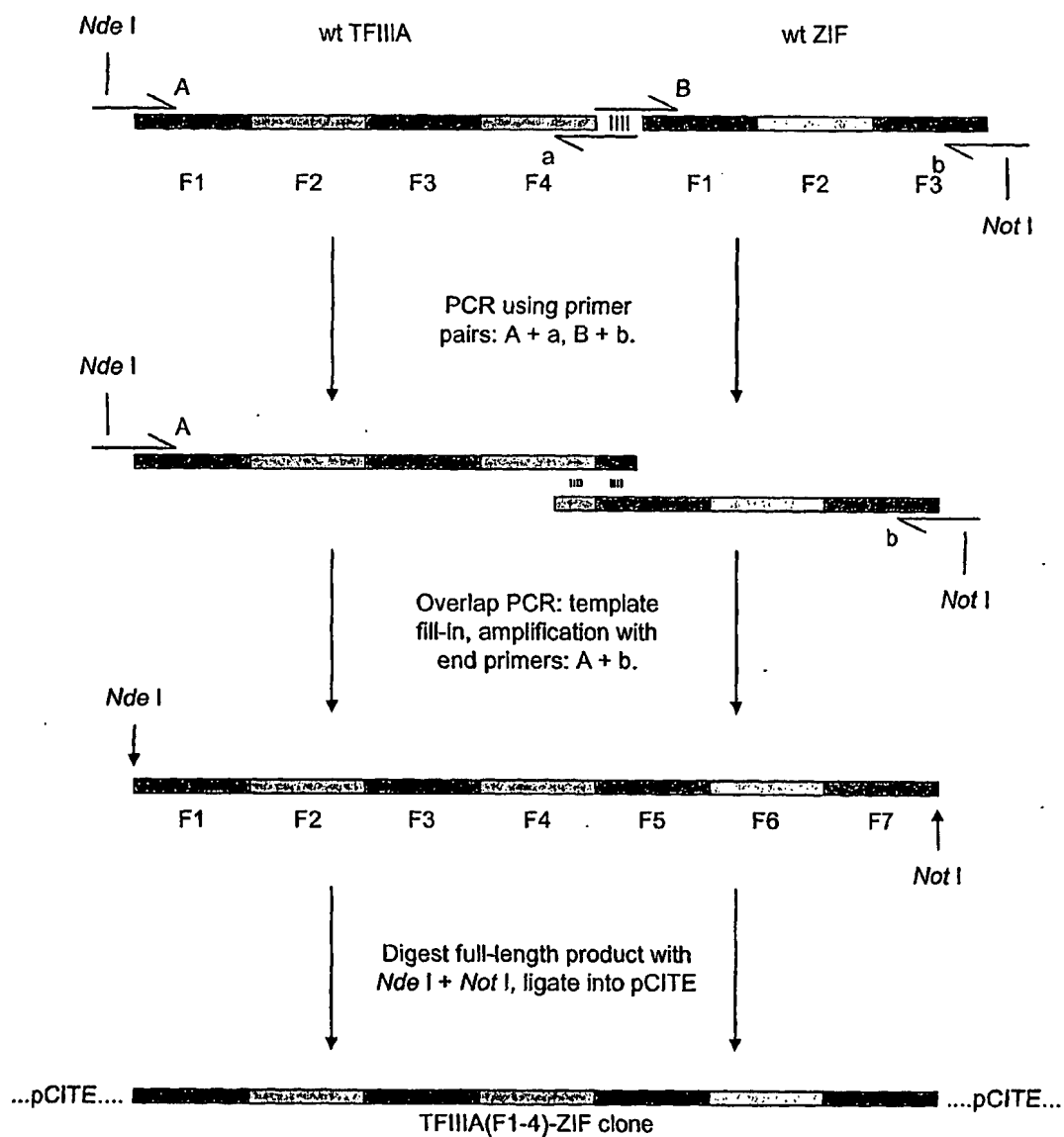


FIG. 13

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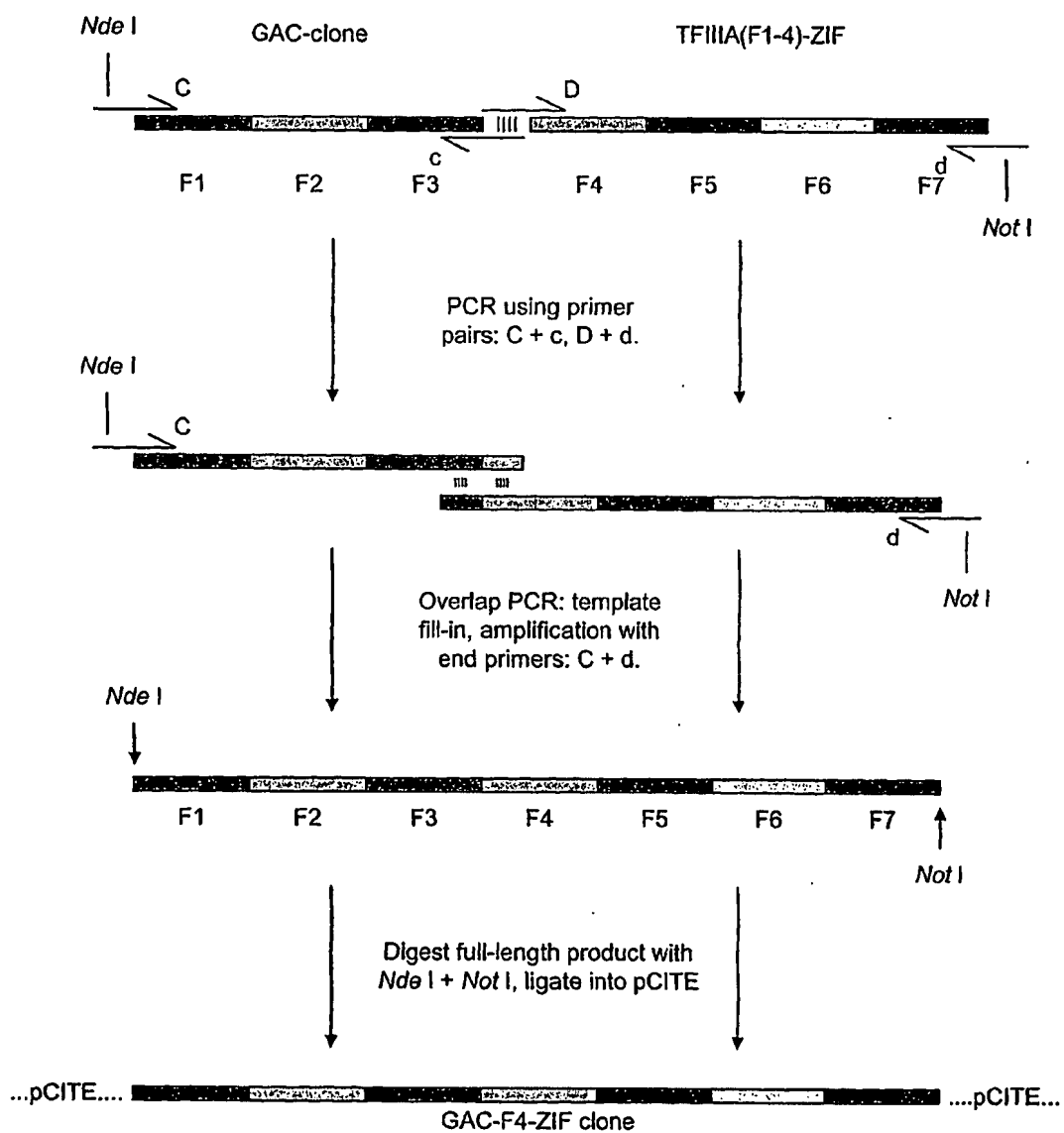


FIG. 14

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TFIIIA(F1-4)-ZIF

SEQ ID NO: 53

```

1/1                               31/11
ATG GGA GAG AAG GCG CTG CCG GTG GTG TAT AAG CGG TAC ATC TGC TCT TTC GCC GAC TGC
M   G   E   K   A   L   P   V   V   Y   K   R   Y   I   C   S   F   A   D   C
61/21                             91/31
GGC GCT GCT TAT AAC AAG AAC TGG AAA CTG CAG GCG CAT CTG TGC AAA CAC ACA GGA GAG
G   A   A   Y   N   K   N   W   K   L   Q   A   H   L   C   K   H   T   G   E
121/41                           151/51
AAA CCA TTT CCA TGT AAG GAA GAA GGA TGT GAG AAA GGC TTT ACC TCG CTT CAT CAC TTA
K   P   F   P   C   K   E   E   G   C   E   K   G   F   T   S   L   H   H   L
181/61                           211/71
ACC CGC CAC TCA CTC ACT CAT ACT GGC GAG AAA AAC TTC ACA TGT GAC TCG GAT GGA TGT
T   R   H   S   L   T   H   T   G   E   K   N   F   T   C   D   S   D   G   C
241/81                           271/91
GAC TTG AGA TTT ACT ACA AAG GCA AAC ATG AAG AAG CAC TTT AAC AGA TTC CAT AAC ATC
D   L   R   F   T   T   K   A   N   M   K   K   H   F   N   R   F   H   N   I
301/101                          331/111
AAG ATC TGC GTC TAT GTG TGC CAT TTT GAG AAC TGT GGC AAA GCA TTC AAG AAA CAC AAT
K   I   C   V   Y   V   C   H   F   E   N   C   G   K   A   F   K   K   H   N
361/121                          391/131
CAA TTA AAG GTT CAT CAG TTC AGT CAC ACA CAG CAG CTG CCG TAT GCT TGC CCT GTC GAG
Q   L   K   V   H   Q   F   S   H   T   Q   Q   L   P   Y   A   C   P   V   E
421/141                          451/151
TCC TGC GAT CGC CGC TTT TCT CGC TCG GAT GAG CTT ACC CGC CAT ATC CGC ATC CAC ACA
S   C   D   R   R   F   S   R   S   D   E   L   T   R   H   I   R   I   H   T
481/161                          511/171
GGC CAG AAG CCC TTC CAG TGT CGA ATC TGC ATG CGT AAC TTC AGT CGT AGT GAC CAC CTT
G   Q   K   P   F   Q   C   R   I   C   M   R   N   F   S   R   S   D   H   L
541/181                          571/191
ACC ACC CAC ATC CGC ACC CAC ACA GGC GAG AAG CCT TTT GCC TGT GAC ATT TGT GGG AGG
T   T   H   I   R   T   H   T   G   E   K   P   F   A   C   D   I   C   G   R
601/201                          631/211
AAG TTT GCC AGG AGT GAT GAA CGC AAG AGG CAT ACC AAA ATC CAT TTA AGA CAG AAG GAC
K   F   A   R   S   D   E   R   K   R   H   T   K   I   H   L   R   Q   K   D

```

FIG. 15

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GAC-F4-ZIF

SEQ ID NO: 54

1/1 31/11
ATG GCA GAA CGC CCG TAT GCT TGC CCT GTC GAG TCC TGC GAT CGC CGC TTT TCT CGC TCG
M A E R P Y A C P V E S C D R R F S R S

61/21 91/31
GAT GAG CTT ACC CGC CAT ATC CGC ATC CAC ACA GGC CAG AAG CCC TTC CAG TGT CGA ATC
D E L T R H I R I H T G Q K P F Q C R I

121/41 151/51
TGC ATG CGT AAC TTC AGT GAT AGA AGC AAT CTT GAA CGT CAC ACG AGG ACC CAC ACA GGC
C M R N F S D R S N L E R H T R T H T G

181/61 211/71
GAG AAG CCT TTT GCC TGT GAC ATT TGT GGG AGG AAG TTT GCC AGG AGT GAT GAA CGC AAG
E K P F A C D I C G R K F A R S D E R K

241/81 271/91
AGG CAT ACC AAA ATC CAT TTA AGA CAG AAG GAC AAC ATC AAG ATC TGC GTC TAT GTG TGC
R H T K I H L R Q K D N I K I C V Y V C

301/101 331/111
CAT TTT GAG AAC TGT GGC AAA GCA TTC AAG AAA CAC AAT CAA TTA AAG GTT CAT CAG TTC
H F E N C G K A F K K H N Q L K V H Q F

361/121 391/131
AGT CAC ACA CAG CAG CTG CCG TAT GCT TGC CCT GTC GAG TCC TGC GAT CGC CGC TTT TCT
S H T Q Q L P Y A C P V E S C D R R F S

421/141 451/151
CGC TCG GAT GAG CTT ACC CGC CAT ATC CGC ATC CAC ACA GGC CAG AAG CCC TTC CAG TGT
R S D E L T R H I R I H T G Q K P F Q C

481/161 511/171
CGA ATC TGC ATG CGT AAC TTC AGT CGT AGT GAC CAC CTT ACC ACC CAC ATC CGC ACC CAC
R I C M R N F S R S D H L T T H I R T H

541/181 571/191
ACA GGC GAG AAG CCT TTT GCC TGT GAC ATT TGT GGG AGG AAG TTT GCC AGG AGT GAT GAA
T G E K P F A C D I C G R K F A R S D E

601/201 631/211
CGC AAG AGG CAT ACC AAA ATC CAT TTA AGA CAG AAG GAC
R K R H T K I H L R Q K D

FIG. 16

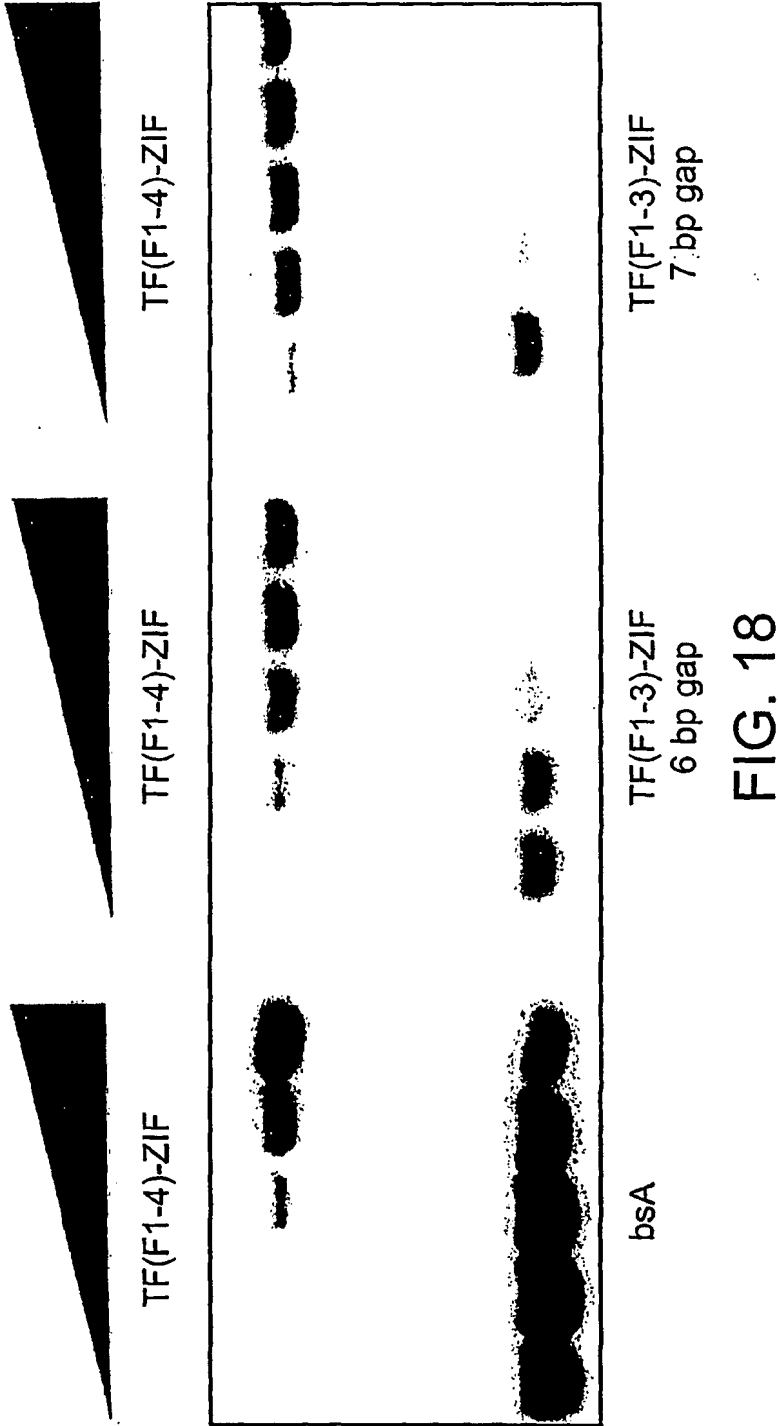
17/27

ZIF-ZnF-GAC

SEQ ID NO: 55

1/1 31/11
 ATG GCA GAA CGC CCG TAT GCT TGC CCT GTC GAG TCC TGC GAT CGC CGC TTT TCT CGC TCG
 M A E R P Y A C P V E S C D R R F S R S
 61/21 91/31
 GAT GAG CTT ACC CGC CAT ATC CGC ATC CAC ACA GGC CAG AAG CCC TTC CAG TGT CGA ATC
 D E L T R H I R I H T G Q K P F Q C R I
 121/41 151/51
 TGC ATG CGT AAC TTC AGT CGT AGT GAC CAC CTT ACC ACC CAC ATC CGC ACC CAC ACA GGC
 C M R N F S R S D H L T T H I R T H T G
 181/61 211/71
 GAG AAG CCT TTT GCC TGT GAC ATT TGT GGG AGG AAG TTT GCC AGG AGT GAT GAA CGC AAG
 E K P F A C D I C G R K F A R S D E R K
 241/81 271/91
 AGG CAT ACC AAA ATC CAT ACC GGT GAA CGG CCG TTC CAG TGT CGA ATC TGC ATG CGT AAC
 R H T K I H T G E R P F Q C R I C M R N
 301/101 331/111
TTC AGT TCT AGT AGC TCT CTT ACC AGC CAC ATC CGC ACC CAC ACA GGT GAG CGG CCG TAT
 F S S S S S L T S H I R T H T G E R P Y
 361/121 391/131
 GCT TGC CCT GTC GAG TCC TGC GAT CGC CGC TTT TCT CGC TCG GAT GAG CTT ACC CGC CAT
 A C P V E S C D R R F S R S D E L T R H
 421/141 451/151
 ATC CGC ATC CAC ACA GGC CAG AAG CCC TTC CAG TGT CGA ATC TGC ATG CGT AAC TTC AGT
 I R I H T G Q K P F Q C R I C M R N F S
 481/161 511/171
 GAT AGA AGC AAT CTT GAA CGT CAC ACG AGG ACC CAC ACA GGC GAG AAG CCT TTT GCC TGT
 D R S N L E R H T R T H T G E K P F A C
 541/181 571/191
 GAC ATT TGT GGG AGG AAG TTT GCC AGG AGT GAT GAA CGC AAG AGG CAT ACC AAA ATC CAT
 D I C G R K F A R S D E R K R H T K I H
 601/201
 TTA AGA CAG AAG GAC
 L R Q K D

FIG. 17



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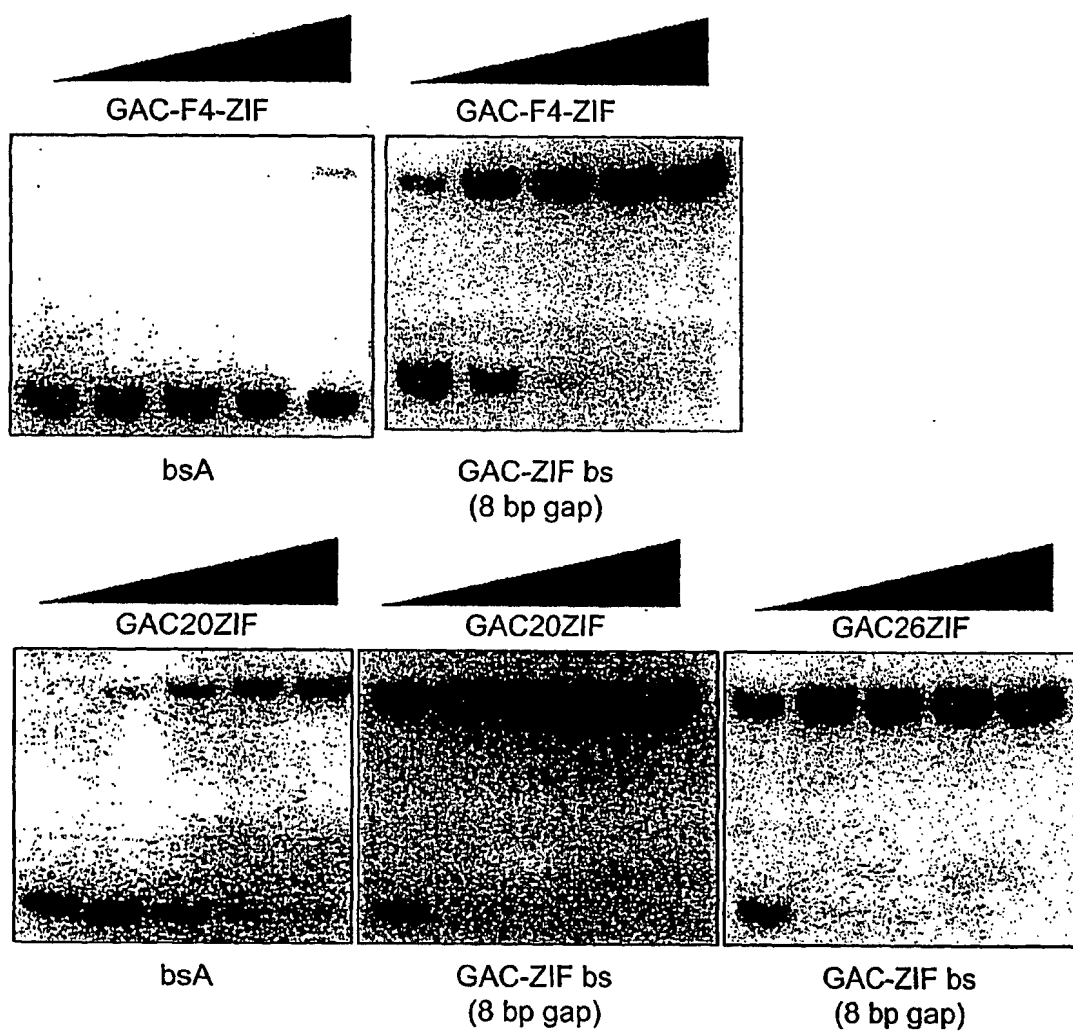
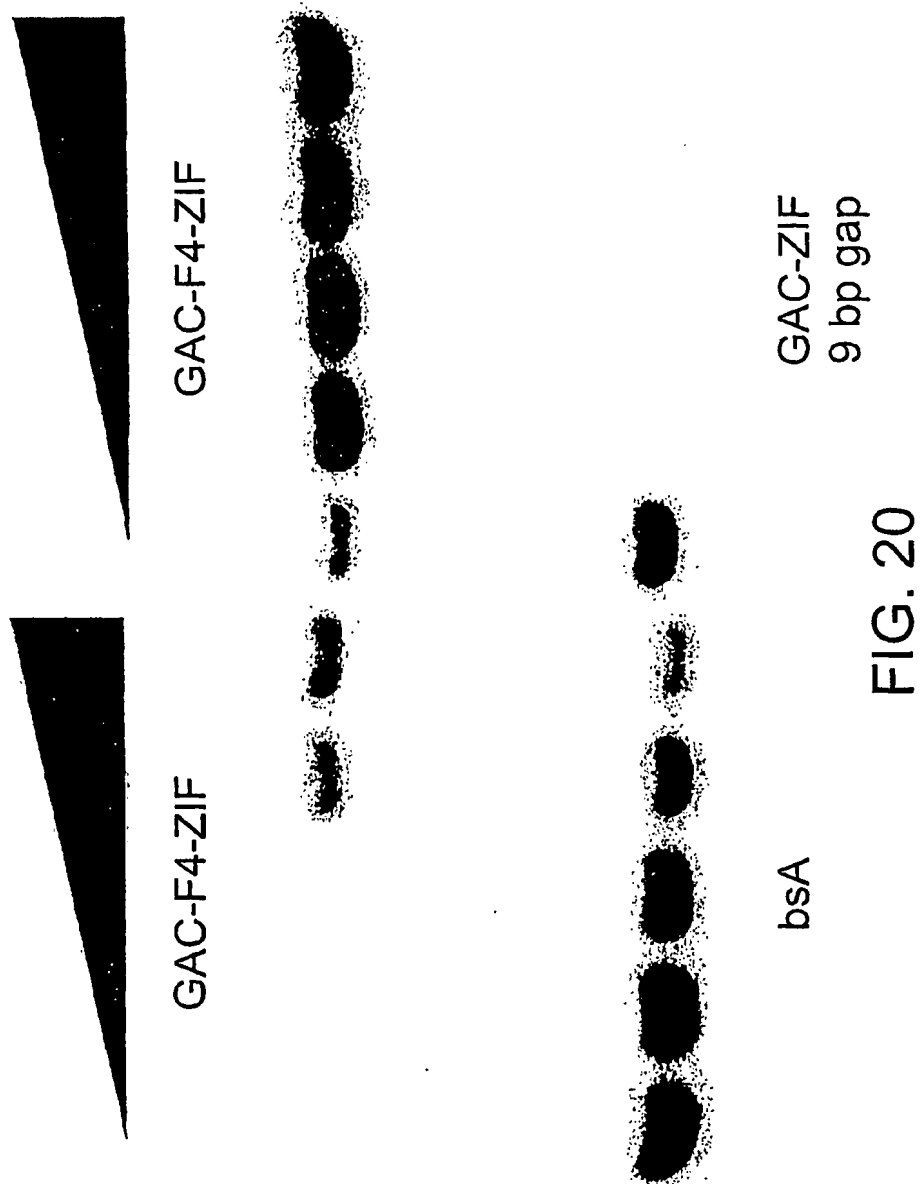


FIG. 19

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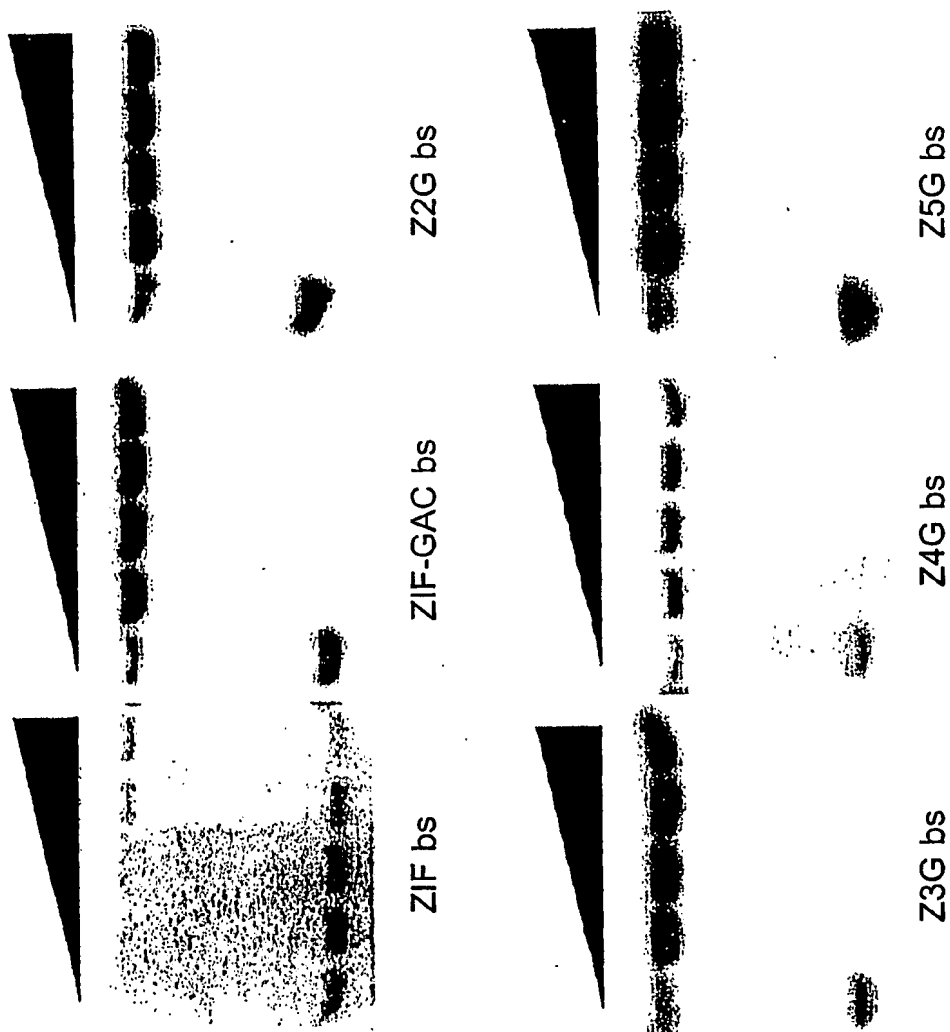


FIG. 21

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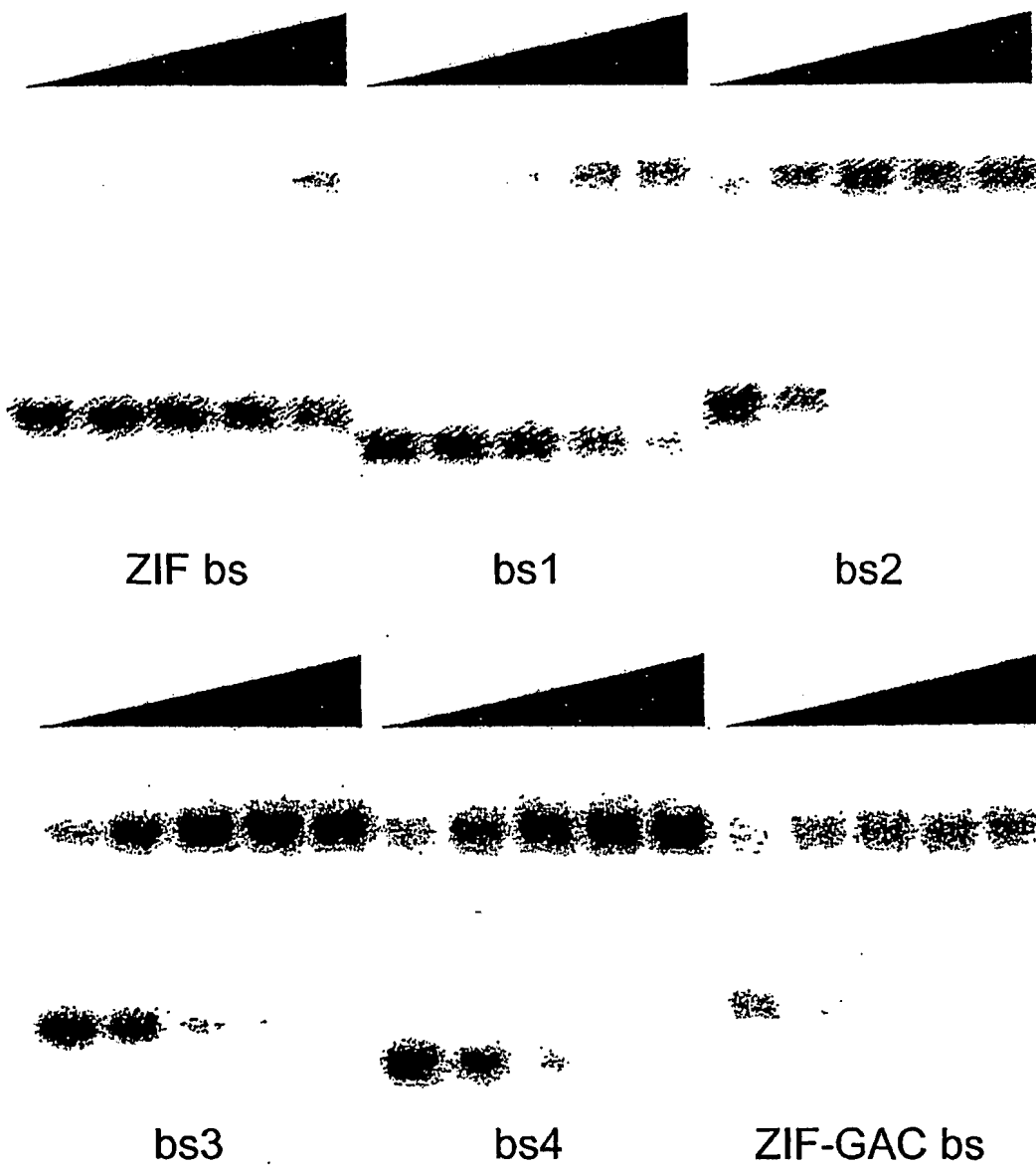


FIG. 22

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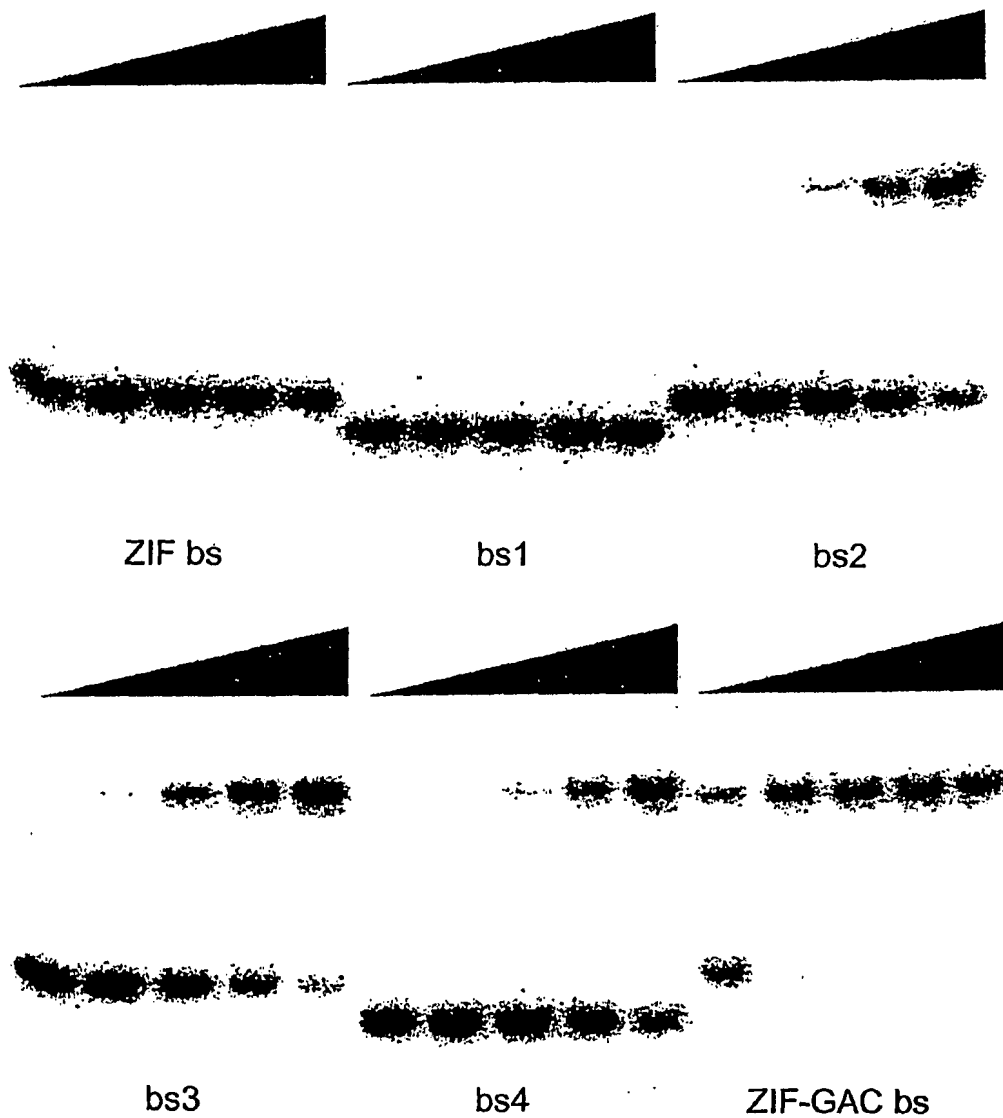


FIG. 23

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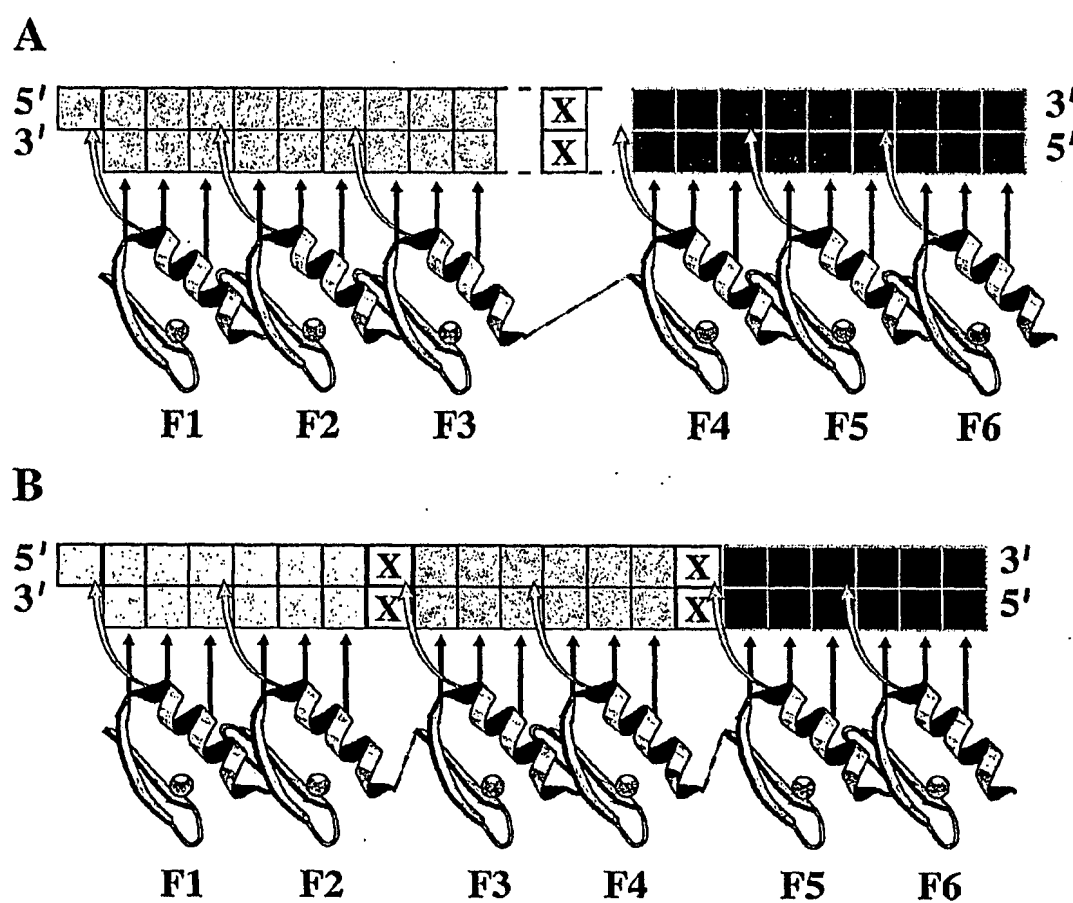
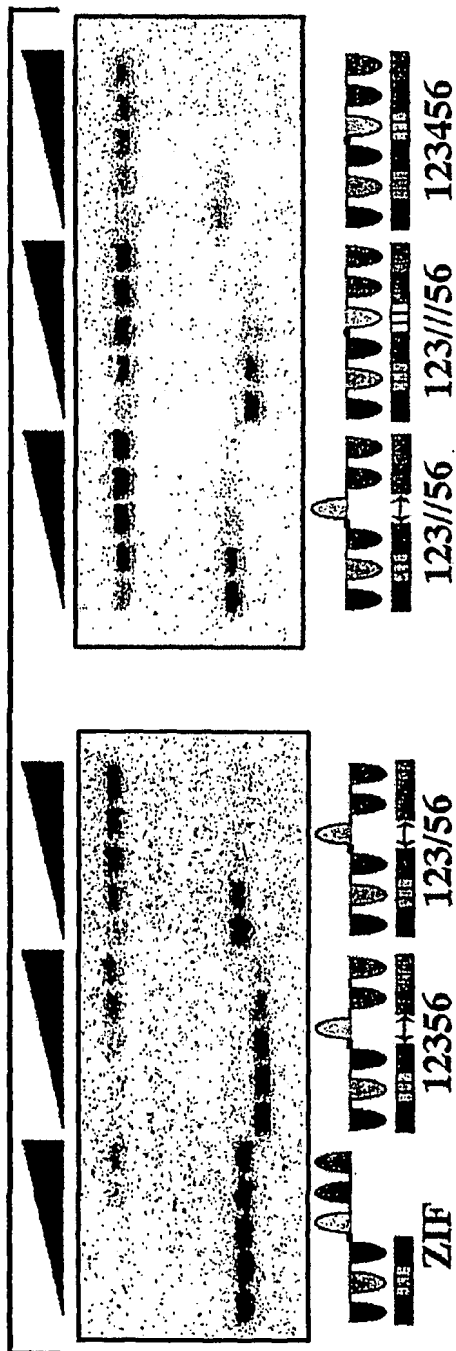


FIG. 24

2x3F ZIF - GAC



3x2F ZGS

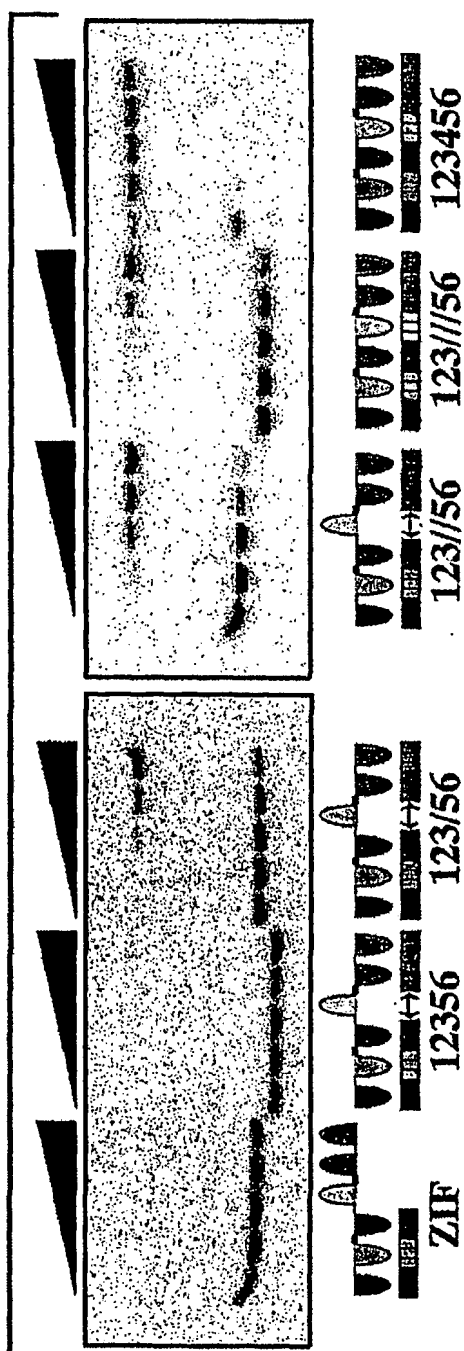


FIG. 25

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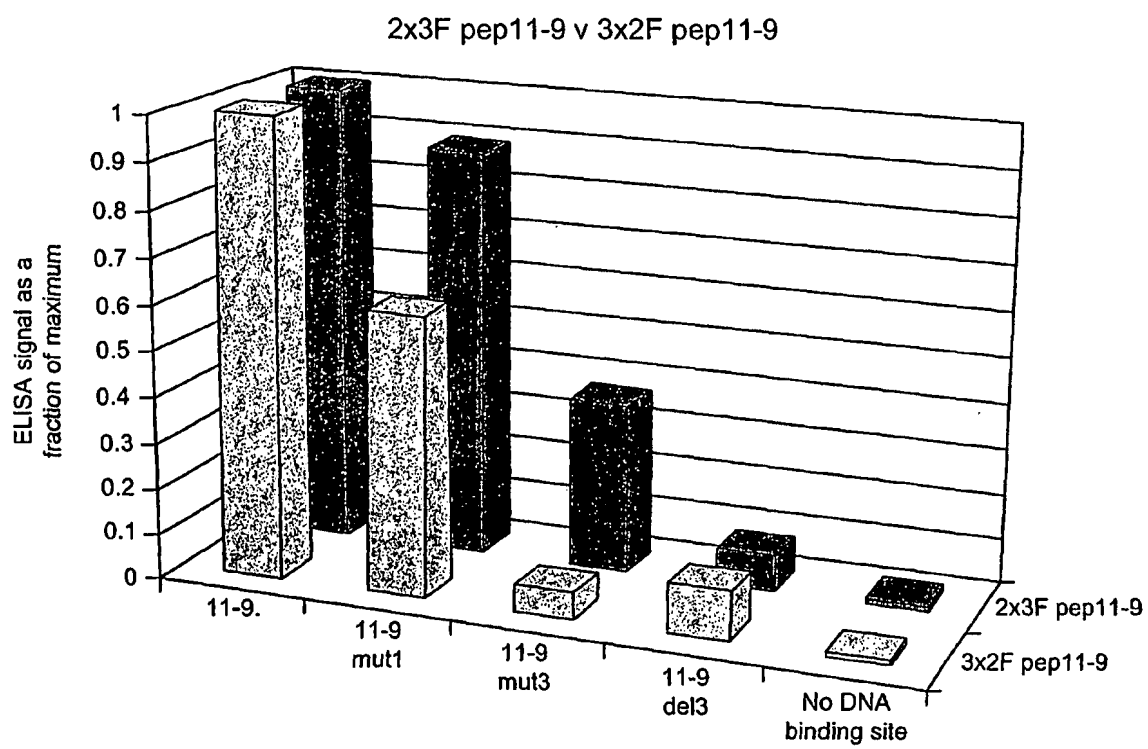
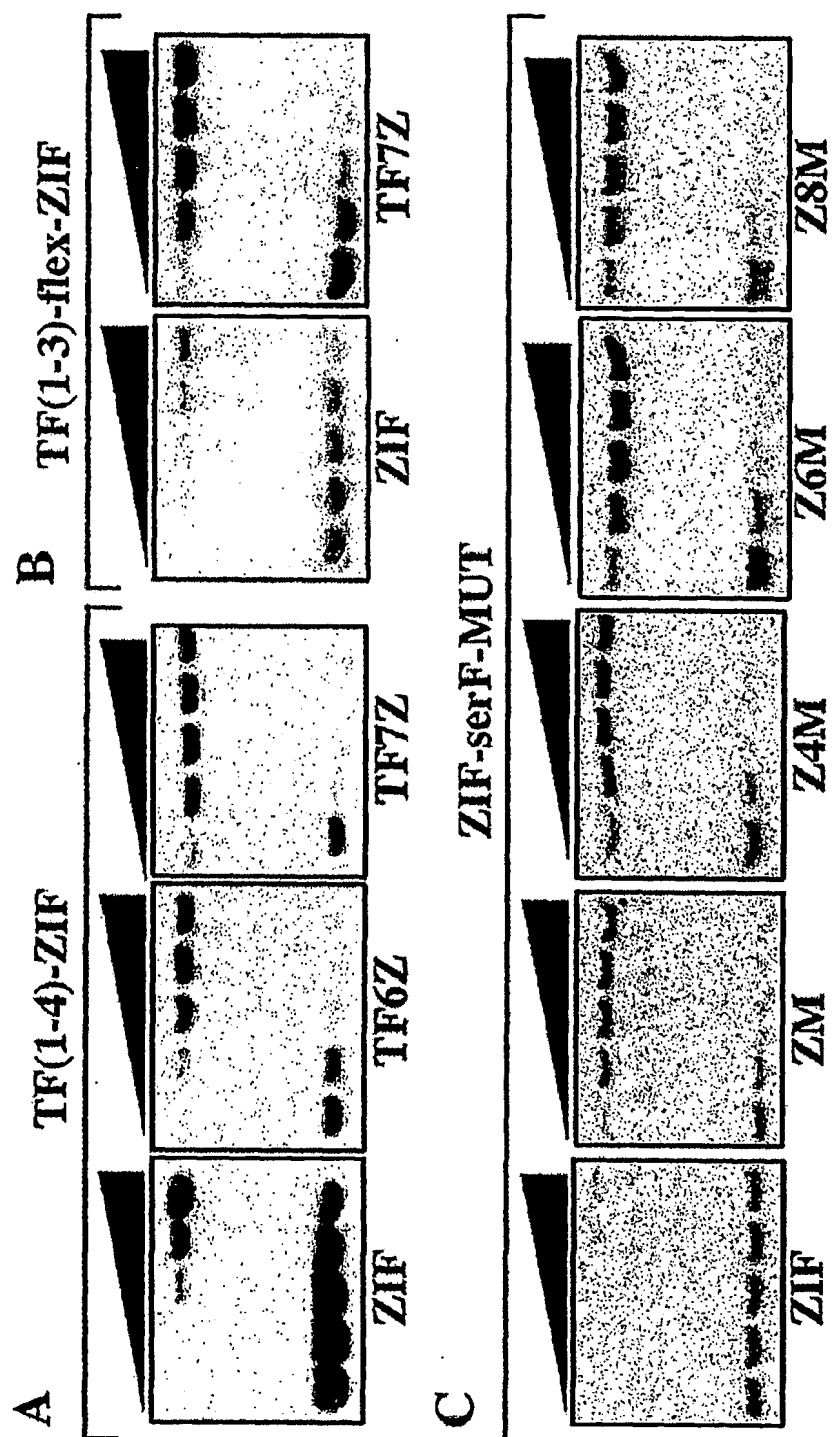


FIG. 26



INTERNATIONAL SEARCH REPORT

International Application No.

PCT/GB 01/00202

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/10 C12N15/12 C12N15/62 C12N15/70 C12N1/21
C07K14/47 C12Q1/68 A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C12Q C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

WPI Data, PAJ, CAB Data, STRAND, EPO-Internal, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>WO 98 54311 A (BARBAS CARLOS F III ;GOTTESFELD JOEL M (US); SCRIPPS RESEARCH INST) 3 December 1998 (1998-12-03)</p> <p>page 53, line 13 -page 54, line 8; claims 1-50; examples 13,14</p> <p>---</p> <p>-/--</p>	<p>1,2, 4-12, 14-16, 18-23, 26,28, 32, 34-39, 44-46</p>

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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A document defining the general state of the art which is not considered to be of particular relevance

E earlier document but published on or after the international filing date

L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

& document member of the same patent family

Date of the actual completion of the international search

25 May 2001

Date of mailing of the international search report

08/06/2001

Name and mailing address of the ISA

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Hornig, H

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/GB 01/00202

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>WO 95 19431 A (BARBAS CARLOS F III ;GOTTESFELD JOEL M (US); SCRIPPS RESEARCH INST) 20 July 1995 (1995-07-20)</p> <p>page 51, line 9 -page 52, line 5; claims 1-58; examples 12,13</p> <p>---</p>	<p>1,2, 4-12, 14-16, 18-23, 26,28, 32, 34-39, 44-46</p>
X	<p>WO 99 45132 A (KIM JIN SOO ;MASSACHUSETTS INST TECHNOLOGY (US); PABO CARL O (US)) 10 September 1999 (1999-09-10) cited in the application</p> <p>page 11, line 15 -page 12, line 18; claims 1-58; figure 1</p> <p>---</p>	<p>1,2, 4-12, 14-16, 18-23, 26,28, 32, 34-39, 41-46</p>
X	<p>LIU QIANG ET AL: "Design of polydactyl zinc-finger proteins for unique addressing within complex genomes." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 94, no. 11, 1997, pages 5525-5530, XP002918175 1997 ISSN: 0027-8424 cited in the application the whole document</p> <p>---</p>	<p>1,2, 4-12, 14-16, 18-23, 26,28, 32, 34-39, 44-46</p>
X	<p>BEERLI ROGER R ET AL: "Toward controlling gene expression at will: Specific regulation of the erbB-2/HER-2 promoter by using polydactyl zinc finger proteins constructed from modular building blocks." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 95, no. 25, December 1998 (1998-12), pages 14628-14633, XP002924795 Dec., 1998 ISSN: 0027-8424 the whole document</p> <p>---</p>	<p>1,2, 4-12, 14-16, 18-23, 26,28, 32, 34-39, 41-46</p>

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 01/00202

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KIM JIN-SOO ET AL: "Getting a handhold on DNA: Design of poly-zinc finger proteins with femtomolar dissociation constants." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 95, no. 6, 17 March 1998 (1998-03-17), pages 2812-2817, XP002168274 March 17, 1998 ISSN: 0027-8424 cited in the application the whole document	1,2, 4-12, 14-16, 18-23, 26,28, 32, 34-39, 44-46
X	WO 96 32475 A (UNIV WASHINGTON ;CHENG CHENG (CA)) 17 October 1996 (1996-10-17) cited in the application page 13, line 9 - line 19; claims 1-32	1,2,6,7, 11,15, 16,18, 19, 21-23, 26,33-39
X	WO 99 42474 A (GENOME DYNAMICS INC) 26 August 1999 (1999-08-26) the whole document	1-3, 11-22, 26,34-39
X	WO 98 53057 A (MEDICAL RES COUNCIL ;ISALAN MARK (GB); CHOO YEN (GB); KLUG AARON ()) 26 November 1998 (1998-11-26) cited in the application claims 1-25	49-51
X	WO 99 47656 A (MEDICAL RES COUNCIL ;ISALAN MARK (GB); CHOO YEN (GB)) 23 September 1999 (1999-09-23) claims 5-10; figure 1	49
A	WO 96 20951 A (MASSACHUSETTS INST TECHNOLOGY ;PABO CARL O (US); SHARP PHILLIP A ()) 11 July 1996 (1996-07-11) claims 1-51; figure 1	

-/--

INTERNATIONAL SEARCH REPORT

itional Application No

/GB 01/00202

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>SEGAL DAVID J ET AL: "Toward controlling gene expression at will: Selection and design of zinc finger domains recognizing each of the 5'-GNN-3' DNA target sequences."</p> <p>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 96, no. 6, 16 March 1999 (1999-03-16), pages 2758-2763, XP002168275</p> <p>March 16, 1999</p> <p>ISSN: 0027-8424</p> <p>cited in the application</p> <p>the whole document</p> <p>---</p>	
P,X	<p>WO 00 23464 A (NOVARTIS ERFIND VERWALT GMBH ;NOVARTIS AG (CH); SCRIPPS RESEARCH I) 27 April 2000 (2000-04-27)</p> <p>page 19, line 13 - line 22; claims 1-21</p> <p>---</p>	<p>1,2,</p> <p>4-12,</p> <p>14-16,</p> <p>18-23,</p> <p>26,28,</p> <p>32,</p> <p>34-39,</p> <p>44-46</p>
T	<p>MOORE MICHAEL ET AL: "Design of polyzinc finger peptides with structured linkers."</p> <p>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 98, no. 4, 13 February 2001 (2001-02-13), pages 1432-1436, XP002168276</p> <p>February 13, 2001</p> <p>ISSN: 0027-8424</p> <p>the whole document</p> <p>---</p>	
T	<p>MOORE MICHAEL ET AL: "Improved DNA binding specificity from polyzinc finger peptides by using strings of two-finger units."</p> <p>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 98, no. 4, 13 February 2001 (2001-02-13), pages 1437-1441, XP002168277</p> <p>February 13, 2001</p> <p>ISSN: 0027-8424</p> <p>the whole document</p> <p>-----</p>	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 01/00202

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
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